INVESTIGATION OF POSSIBLE FACTORS THAT INFLUENCE THE DEVELOPMENT OF NON-CARBON DIOXIDE- DEPENDENT CELLS OF *BRUCELLA ABORTUS* FROM CARBON DIOXIDE-DEPENDENT ONES

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Hamza Keskintepe

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This is to certify that the

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INVESTIGATION OF POSSIBLE FACTORS THAT INFLUENCE THE DEVELOPMENT OF NON-CARBON DIOXIDE-DEPENDENT CELLS OF BRUCELLA ABORTUS FROM CARBON DIOXIDE-DEPENDENT CELLS

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Hamza Keskintepe

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H. J. Stafseth

Major professor

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AN ABSTRACT

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The main objective of this study was to determine the effect of glucose and pH of various mediums on the growth of carbon dioxide-dependent strains of Br. abortus in a normal atmosphere.

Bacto-tryptose and M-peptone agar were the base mediums employed. The strains used throughout the experiments were cultures of carbon dioxide-dependent Br. abortus.

Killed Br. abortus strain 2308 and yeast extract were used as growth promoting factors in tryptose agar for strain 3029.

The colony plating method was employed in order to demonstrate the presence of aerobic cells of Br. abortus which require carbon dioxide. As a source of inoculum for these plates M-peptone agar slants were streaked with cells from the stock culture and incubated for 48 hours at 37° C. in an atmosphere of 5 percent carbon dioxide.

The brucella cells from the M-peptone agar slants were suspended in diluting fluid which was composed of 0.05 percent Bacto-tryptose and 0.5 percent sodium chloride in distilled water. The suspension was adjusted to a standard turbidity of 28 as measured by the photoreflectometer. One ml of the suspension contained 1.5×10^9 cells. The plates were inoculated with one ml of suspension, and incubated, aerobically at 37° C. for four days. At the end of a four-day incubation period, the colonies on each agar medium were

counted and the size of the colonies was measured. Form of growth and colonial type was determined by Huddleson's method (18).

The two mediums used were generally inadequate for the cultivation and isolation of Br. abortus in the absence of carbon dioxide. The absence of carbon dioxide (5%) had bacteriostatic rather than bacteriocidal effect upon naturally occurring strains of Br. abortus.

Tryptose-agar medium, containing 0.1 percent glucose as a source of carbon and energy effected better development of non-carbon dioxide-dependent cells of Br. abortus at pH 7.2. When glucose was increased from 0.1 percent to 0.5 percent in tryptose agar the number of non-carbon dioxide-dependent colonies of Br. abortus was not increased.

Strain 3039 showed that the loss of the requirement for increased carbon dioxide was gradual and occurred in individual cells rather than in all the cells in the culture.

It is not possible to state from the data presented that one medium supports the development of non-carbon dioxide-dependent cells of Br. abortus significantly better than the other mediums.

Experiments concerning the pH of the medium showed each strain under investigation to yield a few colonies at any pH. In this connection, differences in population (from 1 to 8 colonies) are associated with differences in pH or the various mediums. The results showed that a larger number of

colonies may be obtained at pH 7.2 on all mediums in question than at any other pH.

The size of colonies varied from 0.2 mm to 2 mm and all were of the smooth type. In general, the colonies of largest size (2 mm) were developed at pH 7.2. No dissociation to non-smooth colonial types occurred during incubation at 37° C. for four days.

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INTRODUCTION

It has been conclusively demonstrated that carbon dioxide is a vital factor in the growth of practically all bacteria. In their natural environment, bacteria are constantly being exposed to carbon dioxide. However, the growth of some fastidious bacteria is greatly increased by small amounts of carbon dioxide in addition to that found in the atmosphere.

The Brucella is one of those organisms which are characterized by their microaerophilic properties. Particularly the recently isolated Brucella abortus, if it is a naturally occurring one, needs 5 percent carbon dioxide for growth.

Since Huddleson (3) found that carbon dioxide is an essential factor in the isolation and cultivation of Br. abortus, much work by various investigators has been done to explain the metabolic basis for the increased carbon dioxide requirement.

So far, the experimental evidence has been inadequate to offer a definite solution to the problem of carbon dioxide requirement. However, knowledge of the physiological and nutritional requirements of Brucella is essential in attacking many of the problems which are encountered.

The main objective of the present study was to develop non-carbon dioxide-dependent cells of Br. abortus in tryptose agar and M-peptone agar, at various pH levels and with dextrose present.

REVIEW OF LITERATURE

When Bang (1897) first isolated Brucella abortus from the aborted fetuses of cows, it failed to grow aerobically. This problem was overcome by transplanting it many times on artificial culture media. Bang thought the chief factor responsible for this was an oxygen pressure above or below that of the atmosphere (4).

Nowak (13) in 1908, observed that the primary isolation of Br. abortus was greatly enhanced when a culture of Bacillus subtilis was set in a closed jar. He attributed the success of his technique to the reduced oxygen tension in the cultural environment.

Huddleson (3) in 1920, made a study of the atmosphere in tubes containing agar slant cultures of B. subtilis and Br. abortus. This study showed that the growth of Br. abortus was due to an increase in carbon dioxide given off by B. subtilis, instead of decreased oxygen tension as formerly believed.

Huddleson (3) also found that increased carbon dioxide tension was an important factor in the successful isolation of the organism. The correct percentage, by volume, necessary for the growth of Br. abortus was found to be 10 percent.

Valley (18) clearly showed that carbon dioxide, even in small amounts, was necessary for the growth and activities of bacteria, instead of being merely a waste product.

Wilson (19) in a study of the growth of Br. abortus in sealed tubes, proved that carbon dioxide was present as a result of flaming the cotton-wool plug and the paraffined or rubber corks. He confirmed the results of Huddleson showing that under raised pressure of oxygen from 40 to 100 percent no growth occurred unless carbon dioxide was present.

In another paper Wilson (20) demonstrated that growth occurred under any pressure of oxygen from 0.5 percent to 99 percent, when furnished a minimum carbon dioxide (0.5%). Good growth was obtained in any increased carbon dioxide tension from 0.5 percent to 98 percent when furnished a minimum of oxygen (0.5%). He concluded that, in order to obtain maximal growth of Br. abortus, the atmosphere should contain 20 percent oxygen and 5 to 10 percent carbon dioxide.

Wilson (21) also found that sodium bicarbonate could not be substituted for added carbon dioxide in a solid medium, whereas, when bicarbonate solid medium was subjected to carbon dioxide (5-10%) growth occurred.

Studying the gaseous requirement of Br. abortus in semisolid medium Zobell and Meyer (23) reported that growth could be obtained by adding 0.1 percent sodium bicarbonate at either pH 6.8 or 7.2, however, when atmospheric carbon

dioxide was absorbed by potassium hydroxide, in complete absence of carbon dioxide and carbonate, no growth was obtained.

Schuhardt et al. (16) found that certain lots of Difco tryptose had a bactericidal effect on the relatively large inocula of each of 13 cultures of Br. abortus tested.

Zobell and Meyer (23), utilizing a synthetic medium, found that three species of Brucella showed the best growth between pH 6.6 - 7.4. The optimum osmotic pressure for the cultivation of Brucella was from 2 to 6 atmospheres.

Kerby (6) reported that addition of nicotinic acid (30 mg/L) and thiamin (25 mg/L) to Bacto-tryptose agar enhanced the size of colonies of several strains of Br. abortus.

Koser, Breslove and Dorfman (7), studying the accessory growth factor of the brucella group in a chemically defined medium, stated that growth of seven of the eight strains was successfully cultured. The medium was composed of amino acids, glucose and inorganic salts. Thiamin, biotin, nicotinic acid, calcium pantothenate were accessory growth factors for these strains. Thiamin and nicotinamide were required by all the strains, biotin was essential for the Br. abortus strains.

McCullough and Dick (10) attempted to grow a group of recently isolated strains of Br. abortus using the

medium described by Koser (7) and found that carbon dioxide sensitive strains failed to grow in the same synthetic medium with the same accessory growth factors even with increased carbon dioxide tension. After becoming adapted to grow aerobically, 30 of the 41 original stfains grew in the basal medium plus the four accessory growth factors. Failure to grow carbon dioxide sensitive strains of Br. abortus was explained as follows:

1) A lack of unknown accessory factors needed by these strains.

2) Inappropriate constituents employed in the basal medium.

3) An unfavorable electrical potential of the medium.

Ajl and Werkman (1) showed that in the metabolism of Escherichia coli or Aerobacter aerogenes, certain compounds related to the Krebs cycle or their metabolic products can be substituted for carbon dioxide. When synthetic medium was aerated with carbon dioxide, free air growth was poor or absent. When ordinary air was bubbled through the medium good growth took place. Growth was obtained in the absence of carbon dioxide when alphaketoglutaric acid, oxalic acid, glutamic acid were added individually.

Gerhard et al. (2) attempted to grow carbon dioxide-dependent strains of Br. abortus in tryptose broth and chemically defined mediums without an increased atmosphere

of carbon dioxide. Glutamic acid in amounts of 0.1 percent did not substitute for an increased carbon dioxide tension, even though glutamic acid was readily utilized by Brucella. When added to the chemically defined basal medium as nitrogen source, L-glutamic acid again was ineffective. The compounds tested were aspartic acid, arginine, proline, malic acid, fumaric acid, succinic acid, and alphaketoglutaric acid. The results were negative. The controls, incubated in an atmosphere containing 10 percent carbon dioxide, showed good growth.

Sanders and Huddleson (14), studying the influence of atmospheric gases on the multiplication of Brucella, found that there was a demand for large amounts of oxygen by Brucella. Br. abortus exhibited a much greater tolerance for high concentration of carbon dioxide than Brucella suis and Brucella melitensis.

Sanders and Huddleson (15), studying the influence of oxygen on the metabolic activities of Brucella, found that Br. abortus in one percent tryptose medium decomposed more glucose in stagnant air than in an atmosphere of oxygen. The slow rate of multiplication of Br. abortus in that medium exposed to oxygen indicated that the nutrient and environment were not favorable for metabolism. One of the important effects produced by oxygen was an increase in the activity of the oxidative enzyme system that is responsible for the decomposition of glucose by all three of the species.

McAlpine *et al.* (8) reported that in a liquid medium without glucose all strains of *Brucella* produced large amounts of free ammonia. In the medium containing glucose, strains which utilized the carbohydrates produced only slight amounts of ammonia. They were unable to demonstrate the utilization of glucose by *Br. abortus*.

MATERIALS AND METHODS

Cultures

The strains used, throughout the experiments, were stock cultures of carbon dioxide-dependent Br. abortus, strains 3028, 3029, 3039, 3051, and 119, maintained as part of the collection of the Brucella Laboratory. Each culture was identified as Br. abortus by Huddleson. Strains 3028, 3029, and 3039 were described as typical, and 3051 and 119 as atypical Br. abortus strains. The atypical strains were designated as such in that they require an increased carbon dioxide tension for isolation and subsequent cultivation, and produced H_2S like typical Br. abortus, but failed to grow on agar medium containing either basic fuchsin or thionin.

Preparation of Mediums

In order to make a quantitative study of the effect of pH of the medium and glucose on the viability of the organisms under investigation, the following technique was employed.

Three solid mediums were used, namely Bacto-tryptose agar (Difco Laboratories), 0.5 percent glucose-tryptose agar, and M-peptone agar (Albimi Laboratories). These

mediums were adjusted to pH 6.5, 7.2, 7.6 respectively. Tryptose agar and M-peptone agar have been successfully used for isolation, cultivation and for colonial growth studies.

Each medium was prepared by suspending the ingredients in 300 ml of cold distilled water and heating to dissolve completely. It was then dispensed equally in three flasks and sterilized in the autoclave for 15 minutes at 121° C. The medium in each of the flasks was adjusted to a different pH: one 6.5, one 7.2, and the other 7.6. The pH of the mediums was adjusted to the desired level with 10 percent sodium carbonate and 1/10 N HCl after autoclaving. Sodium carbonate (10%) was filtered through a D-8 Hormann pad as a means of sterilization. The contents were then poured into four Petri plates, thus giving four plates of each pH for each medium. The poured plates were dried in a 37° C. incubator for 48 hours.

Killed Br. abortus strain 2308 and yeast extract were used as growth promoting factors in tryptose agar for strain 3029.

Inoculation of the Mediums

The presence of aerobic cells, among the strains of Br. abortus which require carbon dioxide, can be best demonstrated by employing the colony plating method.

In order that the data obtained throughout the experiments might be comparable, an attempt was made to inoculate the same number of organisms on constant volumes of mediums.

As a source of inoculum for these plates, M-peptone agar slants were streaked with cells from the stock culture and incubated for 48 hours at 37° C. in an atmosphere of 5 percent carbon dioxide. The brucella cells from the M-peptone agar slants were suspended in diluting fluid which was composed of 0.05 percent Bacto-tryptose and 0.5 percent sodium chloride in distilled water. The suspension was thoroughly shaken to achieve complete dispersion of the cells and adjusted to a standard turbidity of 28 as measured by the photoreflectometer. The suspension then contained 1.5×10^9 cells per ml. One ml of the suspension, 1.5×10^9 organisms, was aseptically pipetted on each of four plates of each medium. The plates were then rotated to obtain an even spread of organisms over the surface. The inoculated mediums were incubated, aerobically at 37° C. for four days.

Examination of Plates

Plate count. At the end of a four-day incubation period, the colonies on each agar medium were counted.

Colony size. The size of the colonies that developed on each medium was measured on the fourth day of incubation.

Form of growth and colonial type was determined by Huddleson's method (18):

a) Color

b) Consistency and texture

c) Acriflavine spot test

d) Staining with crystal violet.

RESULTS

A. The growth of five carbon dioxide-dependent strains of Br. abortus was studied on three agar mediums in a normal atmosphere.

Tables I, II, III, and IV show the number and size of the colonies which developed from cells of strains 3028, 3029, 3051, and 119 after incubation for four days at 37° C. in a normal atmosphere. All four strains gave rise to very few colonies on all mediums. The size of the colonies which developed on each medium, varied in diameter, and is indicated as one plus to five plus and measured approximately 0.2 mm to 2 mm on the fourth day of the incubation period.

The data in Table I show that Br. abortus strain 3029, incubated 4 days, gave rise to one or two colonies at pH 6.5 to 7.2 and no colonies at pH 7.6 on each of the mediums. On the fifth day of incubation, the number of colonies on tryptose agar at pH 6.5 had increased to four. All colonies which developed were of the smooth colonial type and their size varied from 0.2 mm to 1 mm. The colonies on Bacto-tryptose agar were larger at pH 6.5 than at pH 7.2 or 7.8.

Table II shows the results obtained with strain 3028. On tryptose agar (0.5% glucose) at pH 5.5, 4, 7, and 8 colonies developed on the fourth, fifth and sixth day of the incubation period respectively, whereas, on the same mediums at pH 7.8 there developed one colony on the fourth day and three colonies on the sixth day of incubation. The size of these colonies was 0.2 to 0.3 mm in diameter. No growth was obtained on M-peptone agar at pH 6.5 and only one colony on tryptose agar at pH 6.5 throughout the incubation period. Larger colonies developed at pH 6.5 and 7.2 in tryptose agar than on any other medium used. All colonies were smooth.

Strains 3051 and 119 are the two atypical forms of Br. abortus. The results obtained are shown in Tables III and IV. In the case of both strains, the largest number and size of colonies were obtained at pH 7.2 on all three mediums employed. No colonies of strain 119 were observed at pH 6.5 on any medium.

B. An experiment was conducted to find out whether killed brucella cells and yeast extract in tryptose agar medium would have any effect on the development of non-carbon dioxide-dependent cells of strain 3029 Br. abortus. For this purpose, a suspension of strain 2308 was added to tryptose agar medium before autoclaving.

By analyzing Tables I and VII comparatively, one may note that certain differences are evident. Strain 3029, on Bacto-tryptose agar at pH 7.2 gave rise to one colony, and none at pH 7.6, whereas, on tryptose agar, containing killed Br. abortus cells 7 colonies developed at pH 7.2 and 4 colonies at pH 7.7. Furthermore, the size of these colonies on the medium containing killed cells was larger than that of the others. Smooth type colonies were observed.

Yeast extract was employed as a growth promoting factor in tryptose agar at concentrations of 0.1 and 1 percent. In this experiment also, strain 3029 of Br. abortus was used. Table IX shows that only one colony developed at pH 7.2 and none at pH 6.5 and 7.6 on both 0.1 and 1 percent yeast-tryptose agar.

C. Strain 3039 had been employed for some early investigations. In these studies, this strain had given rise to only two colonies at pH 7.32 (Table VIII). During the following three months the strain was transferred five or six times on tryptose agar slants and incubated in an atmosphere of 5 percent carbon dioxide. Growth of strain 3029 in a normal atmosphere was then studied again to determine the influence of frequent transfers on the development of non-carbon dioxide-dependent cells. Thirty-seven to 137

colonies were observed on the plates (Table V). This is a marked increase over the two colonies observed in cultures of this strain before the period of frequent transfers and the 1-8 colonies which developed in cultures of the other four strains of Br. abortus not subjected to the period of frequent transfer and growth in 5 percent carbon dioxide.

A larger number of colonies was obtained on the three mediums at pH 7.2 than at pH 6.5 or pH 7.8. Tryptose agar at pH 7.2 effected better development of non-carbon dioxide-dependent colonies than the other mediums under investigation. The size of colonies that developed on each medium varied in diameter. However, pH 7.2 gave rise to the largest colony size (1.7 mm to 2 mm). All colonies were identified as the smooth type.

D. It was estimated that the inoculum (1 ml) placed on each plate contained 1.5×10^9 viable cells. Only a few colonies developed in a normal atmosphere. To determine whether the remaining cells in the original inoculum were killed after incubation at 37° C. for four days, or merely suppressed due to the absence of carbon dioxide, a wire loop was streaked across a clear area of a tryptose agar plate culture of strain 3039 and then streaked on an M-peptone agar slant. Good growth was obtained when this slant was incubated in the presence of 5 percent carbon dioxide for 48

hours. Using this growth, tryptose agar plates were then inoculated with 1.5×10^9 viable cells. The data obtained, recorded in Table VI, show that pH 7.2 gave rise to 49 colonies which were larger than the colonies which developed at pH 6.6 and 7.6. Twenty-five colonies developed at pH 6.6 and 17 colonies at pH 7.6. All colonies were the smooth type.

TABLE I

GROWTH OF CO_2-DEPENDENT STRAIN 3029 IN NORMAL ATMOSPHERE

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3029	Tryptose agar	6.5	6×10^9	2	3+
"	"	7.2	6×10^9	1	1+
"	"	7.6	6×10^9	-	-
3029	Tryptose agar (0.5% glucose)	6.5	6×10^9	1	1+
"	"	7.2	6×10^9	2	2+
"	"	7.6	4.5×10^9	-	-
3029	M-peptone agar	6.5	6×10^9	1	2+
"	"	7.2	4.5×10^9	2	3+
"	"	7.6	6×10^9	-	-

1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

TABLE II

GROWTH OF CO_2-DEPENDENT STRAIN 3028 IN NORMAL ATMOSPHERE

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3028	Tryptose agar	6.5	6×10^9	1	4+
"	"	7.2	4.5×10^9	2	5+
"	"	7.8	6×10^9	2	1+
3028	Tryptose agar (0.5% glucose)	5.5	6×10^9	4	1+
"	"	7.2	6×10^9	2	1+
"	"	7.8	6×10^9	1	2+
3028	M-peptone agar	6.5	6×10^9	-	-
"	"	7.2	6×10^9	1	1+
"	"	7.8	6×10^9	3	2+

1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

TABLE III

GROWTH OF CO_2-DEPENDENT STRAIN 3051 IN NORMAL ATMOSPHERE

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3051	Tryptose agar	6.5	6×10^9	1	1+
"	"	7.2	6×10^9	5	4+
"	"	7.6	6×10^9	2	2+
3051	Tryptose agar (0.5% glucose)	6.7	4.5×10^9	1	1+
"	"	7.2	6×10^9	8	5+
"	"	7.6	6×10^9	7	4+
3051	M-peptone agar	6.6	6×10^9	4	3+
"	"	7.1	6×10^9	6	5+
"	"	7.5	6×10^9	5	3+

1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

TABLE IV

GROWTH OF CO_2-DEPENDENT STRAIN 119 IN NORMAL ATMOSPHERE

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
119	Tryptose agar	6.5	6×10^9	1	1+
"	"	7.2	6×10^9	4	5+
"	"	7.6	6×10^9	-	-
119	Tryptose agar (0.5% glucose)	6.7	6×10^9	2	1+
"	"	7.2	6×10^9	4	5+
"	"	7.6	6×10^9	-	-
119	M-peptone agar	6.6	6×10^9	1	1+
"	"	7.1	6×10^9	3	4+
"	"	7.5	6×10^9	-	-

1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

TABLE V

GROWTH OF CO_2-DEPENDENT STRAIN 3039 IN NORMAL ATMOSPHERE

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3039	Tryptose agar	6.5	6×10^9	112	4+
"	"	7.2	6×10^9	137	5+
"	"	7.8	6×10^9	133	1+
3039	Tryptose agar (0.5% glucose)	5.5	6×10^9	30	1+
"	"	7.2	6×10^9	130	4+
"	"	7.8	6×10^9	108	2+
3039	M-peptone agar	6.5	6×10^9	37	1+
"	"	7.2	4.5×10^9	96	5+
"	"	7.8	4.5×10^9	80	3+

1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

TABLE VI

ADAPTATION OF CARBON DIOXIDE-DEPENDENT STRAIN 3039 TO NORMAL ATMOSPHERE*

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3039	Tryptose agar	6.6	6×10^9	25	4+
"	"	7.2	6×10^9	49	5+
"	"	7.7	6×10^9	17	1+

* Inoculum for seed culture was composed of cells which did not multiply in normal atmosphere.
1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

TABLE VII

GROWTH OF CO_2-DEPENDENT STRAIN 3029 IN TRYPTOSE MEDIUM CONTAINING KILLED CELLS

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3029	Tryptose agar (killed cells of Br.)	6.5	6×10^9	4	2+
"	"	7.2	6×10^9	7	5+
"	"	7.7	6×10^9	4	3+

1) Incubated at 37° C. for 4 days.
2) Colonies were 0.2 - 2 mm.

TABLE VIII

GROWTH OF CO_2-DEPENDENT STRAIN 3039 IN NORMAL ATMOSPHERE BEFORE SERIAL TRANSFERS

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of colonies [1]	Size of Colonies [2]
3039	Tryptose agar	7.32	6×10^9	2	3+
"	"	7.2	6×10^9	3	4+

1) Incubated at 37° C. for 4 days.
2) Colony size : + to 5+ = 0.2 to 2 mm.

TABLE IX

GROWTH OF STRAIN 3029 ON TRYPTOSE-AGAR CONTAINING YEAST-EXTRACT

Strain	Medium	pH	Number of organisms inoculated on four agar plates	Total number of Colonies [1]	Size of Colonies [2]
3029	Tryptose-agar (0.1% yeast-extract)	6.5	6×10^9	-	-
"	"	7.2	6×10^9	1	3+
"	"	7.6	6×10^9	-	-
3029	Tryptose-agar (1% yeast-extract)	6.5	6×10^9	-	-
"	"	7.2	6×10^9	1	4+
"	"	7.6	6×10^9	-	-

1) Incubated at 37° C. for 4 days.
2) Colony size: + to 5+ = 0.2 to 2 mm.

DISCUSSION

This study was initiated in an effort to determine the effect of glucose and pH of various mediums on the growth of carbon dioxide-dependent strain of Br. abortus in a normal atmosphere.

Bacto-tryptose and M-peptone agar were the base mediums employed. Both mediums have been successfully employed in the isolation, cultivation, and in the examination of the colonial growth of Brucella.

An optimum pH is determined by many factors, such as the type of medium, temperature of incubation, rate of growth, and time of observation. Observers have generally come to agree that Brucella shows maximum growth between pH 6.6 to 6.8.

Experiments concerning the pH of the medium showed each strain under investigation to yield a few colonies at any pH. In this connection, differences in population (from 1 to 8 colonies) are associated with differences in pH of the various mediums. The results show that a larger number of colonies may be obtained at pH 7.2 on all three mediums in question than at any other pH.

Bacto-tryptose agar effected the development of a slightly larger number of non-carbon dioxide-dependent colonies than the other agar mediums. M-peptone agar produced almost the same

number and size of colonies at any pH. It is not possible to state from the data presented that one of these two mediums supports the development of non-carbon dioxide-dependent cells of Br. abortus significantly better than the others.

In analyzing the results as a whole, we see that all strains under consideration yielded a smaller number of colonies when the pH of the mediums was above and below 7.2. The diameter of the colonies generally was larger (1.8 mm to 2 mm) at pH 7.2, and, as the pH of all mediums decreased below 7.2, smaller colonies developed. However, neither the number nor the size of the colonies can be based simply on the effect of pH. None of the three mediums, regardless of pH, could be used for the isolation or cultivation of naturally occurring Br. abortus in the absence of increased carbon dioxide tension.

As shown in Table V, the colony counts of Br. abortus strain 3039 differ with respect to the colony counts of the other strains which were investigated. Since this strain had given rise to only two aerobic colonies from 3×10^9 seeded viable cells at pH 7.32 before it was transferred through several culture mediums, one may conclude that Br. abortus strain 3039 had partially lost its requirements for increased carbon dioxide concentration. This observation confirms that of Marr and Wilson (11) who demonstrated that

the loss of increased carbon dioxide requirement begins in individual cells, rather than in all the cells in a culture. The results recorded in Table VI, on one hand prove that the absence of carbon dioxide is bacteriostatic rather than bacteriocidal. On the other hand, the development of these colonies was an indication that individual cells of Br. abortus (strain 3039) had gradually lost their requirement for increased carbon dioxide.

Sanders (15) stated that the metabolism of glucose by all species of Brucella is a rapid oxidation of carbohydrates and that the major end product is carbon dioxide. She also stated that an oxidative enzyme system is responsible for the decomposition of glucose.

Since glucose, the only available source of carbon and energy, is readily utilized for carbon dioxide production by Br. abortus, it was thought that by increasing the amount of glucose from 0.1 percent to 0.5 percent in tryptose agar at various pH's, one might increase the number of non-carbon dioxide-dependent cells. The strain in question gave rise to a higher colony count on 0.5 percent glucose-tryptose agar than on the other mediums under investigation (Tables I, II, III, and IV). But the slightly greater number of colonies on tryptose agar (0.5% glucose) was not sufficient to warrant the conclusion that 0.5 percent glucose is necessary

in tryptose agar for better support of non-carbon dioxide-dependent cells of Brucella.

Heat-killed Br. abortus (strain 2308) was employed as a growth promoting factor in tryptose agar. It exhibited very little effect, if any, upon the number of non-carbon dioxide-dependent cells of Br. abortus. However, it did effect a larger colony size at pH 7.2. This unknown growth-promoting factor is heat stable.

Yeast extract as a growth-promoting factor in tryptose agar did not exhibit any effect upon the number of non-carbon dioxide-dependent cells of Br. abortus (strain 3029). All colonies were identified as smooth type.

Carbon dioxide has been recognized as an essential and vital factor to microbial life. A certain minimal amount of this factor is just as important for bacteria as it is for plants, the higher animals, and man.

Huddleson (3) first proved that carbon dioxide was necessary for the growth of Br. abortus. It is known that the function of carbon dioxide is not that of an added stimulus but that it is directly concerned with the metabolism of the bacterial cell itself. Huddleson, analyzing the work of Marr and Wilson (12), stated that increased amount of carbon dioxide is needed by the cell for the building of protein.

SUMMARY

Tryptose-agar medium, containing 0.1 percent glucose as a source of carbon and energy effected better development of non-carbon dioxide-dependent cells of Br. abortus at pH 7.2 than at pH 6.8 or pH 7.6.

Three mediums which were used were generally inadequate for the cultivation and isolation of Br. abortus in the absence of carbon dioxide.

The absence of carbon dioxide (5%) had a bacteriostatic effect rather than bacteriocidal effect upon naturally occurring strains of Br. abortus.

Strain 3039 showed that the loss of the requirement for increased carbon dioxide was gradual and occurred in individual cells rather than in all the cells in the culture.

When glucose was increased from 0.1 percent to 0.5 percent in tryptose agar the number of non-carbon dioxide-dependent colonies of Br. abortus was not increased.

The size of colonies varied from 0.2 to 2 mm and all were of the smooth type. In general, the colonies (2 mm) of largest size were developed at pH 7.2. No dissociation to non-smooth colonial types occurred during incubation at 37° C. for four days.

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