RELATIVE MERITS OF STANDARD VERSUS MODIFIED PROCEDURES FOR ISOLATION OF *BRUCELLA ABORTUS* FROM MILK

by

DEANE FAY WEBER

B. S., Jamestown College, 1950

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1952

TABLE OF CONTENTS

INTRODUCTION

Since the discovery and isolation of the etiological agent of brucellosis in cattle by Bang, numerous questions concerning this agent and its insidious nature have been asked. It is from these questions that researchers have formulated theories and problems in order to find answers to such inquiries. Much time, work, and patience have been spent on the numerous problems connected with brucellosis and not without result; still, many questions remain unanswered and many problems unsolved. Theobald Smith once said concerning the problem of brucellosis, "The problem has grown more obscure and complex with the publications of fresh cases and the more thorough study of the organisms obtainable from them".

The isolation of Brucella abortus from milk is one of the specific means of detecting Brucella infection, upon which depends the answer to many questions being asked concerning brucellosis. Yet, due to the complexity of the nature of Brucella abortus, the problem of isolation still presents some difficulties. One important unsolved isolation problem is the frequently reported intermittent isolation of Brucella abortus from cows showing a positive Brucella blood agglutination titer.

This intermittent isolation of Brucella abortus from the milk of positive Brucella reactors suggests the possibility of a lack of accuracy or ineffectiveness in present methods of isolation. Therefore, it would be decidedly advantageous if an accurate and delicate method of isolating Brucella abortus from milk

were available. One could then be reasonably certain of the presence or absence of these organisms in milk depending upon his findings. It was with the possibility in mind of improving the delicacy and accuracy of present day methods of isolating Brucella from milk that this project was undertaken.

REVIEW OF LITERATURE

The presence of Bacillus abortus (Brucella abortus) in the milk of cows was foreshadowed in some investigations in 1894 by the Bureau of Animal Industry, Department of Agriculture. In a footnote to experiments by E. C. Schroeder, Theobald Smith, then chief of the Division of Pathology, called attention to the peculiar lesions in a guinea pig caused by the intra-abdominal injections of milk, and warned against its identification with tuberculosis. This peculiar inoculated-disease of guinea pigs remained unidentified until Smith and Fabyan, in 1912, called attention to the great likeness between it and the disease produced by the abortion bacillus, and concluded that the abortion bacillus must have been present in the samples of milk studied in 1893.

Aside from the fact that the inoculation of milk into guinea pigs caused peculiar lesions similar to tuberculosis, but from which no acid-fast organisms were obtained, nothing more was known of the organism until Berhard Bang (1897) and his assistant, Stribolt, found in the uterine exudate of a killed, pregnant cow, showing premonitory symptoms of abortion, a short bacillus, whose

body contained one, two, or three granules taking the stain more readily than the body. The bacilli were easily cultivated in test tubes containing serum gelatine agar; meat-water peptone broth to which had been added 0.75 percent agar and 5 percent gelatine. This was melted and cooled to about 45° C. and then mixed with about half its volume of sterile, blood serum. While still fluid, the medium was inoculated into test tubes, cooled and incubated at 37° C. The colonies developed in a definite zone, lying about 0.5 cm under the surface of the nutrient medium and having a thickness of from 1 to 1.5 cm.

Bang concluded, therefore, that the bacillus is neither anaerobic nor aerobic in the usual sense; but, exhibits a very peculiar behavior in respect to oxygen, requiring a partial pressure of oxygen less than that present in the atmosphere.

Nowak (1908), isolating Brucella abortus from the placenta of an abortion of a heifer, used a serum plate which he developed for the isolation of the abortion bacillus. This consisted of ordinary agar which was melted and cooled to 50° C., then mixed with 25 percent its volume of naturally-sterile, blood serum, poured into sterile petri dishes, and allowed to solidify. The serum-agar plates were incubated in the air for 24 hours and the colonies which developed were then placed in a Novy anaerobe jar, together with one petri dish plate-culture of Bacillus subtilis; the jar was closed and incubated four days at 37° C. Confirmation was by inoculation of a 0.5 cc suspension subcutaneously into a guinea pig.

McNeal and Kerr (1910) were the first in America to isolate Bacillus abortus (Brucella abortus) from the placenta of cows that had aborted. Guinea pig inoculation and Nowak's plate method for isolations and confirmations were used. They concluded that the bacillus of Bang is the microbic cause of at least some of the contagious abortion of cattle in this country.

Melvin (1911), in an introductory statement to BAI Circular 198, stated that Mohler and Traum, working simultaneously with Schroeder and Cotton, definitely settled the question of the identity of the organism which gave similar lesions to tuberculosis from injected milk and the organisms that cause Bang's disease.

Schroeder and Cotton (1911), by inoculating guinea pigs, found the Bacillus abortus (Brucella abortus) to occur in milk and using tissues of guinea pigs, re-isolated Brucella abortus on artificial media, consisting of agar with 6 percent glycerin and over 50 percent ox gall. Colonies were small, pearly, slightly convex, and pale gray.

Mohler and Traum (1911) isolated Bacillus abortus (Brucella abortus) from tissues, notably uterine exudate, of infected cows.

The discovery of Bacillus abortus (Brucella abortus) in the udder by Schroeder and Cotton was corroborated by Smith and Fabyan (1912), who found that the udder is a reservoir for Bacillus abortus (Brucella abortus).

In reporting on the presence of Bacillus abortus (Brucella abortus) in milk, Fabyan (1913) collected milk samples from twelve

cows, two that had aborted and ten at random. Samples were collected after the first milk was rejected, 40 to 50 cc were milked directly into sterile glass tubes held at a slant; these were corked, packed in snow, and placed in the icebox until examination. Cultures were made on slanted agar from both cream and sediment. The slants were incubated for 24 hours at 37° C. The colonies then examined and marked, and reincubated using Nowak's method mentionned on page 3. No other colonies appeared and no Bacillus abortus (Brucella abortus) could be demonstrated by cultural methods. Guinea pigs were inoculated abdominally with cream and cream and sediment mixed, 2.5 to 6 cc used. Two of the guinea pigs had fever, and upon autopsy, had characteristic lesions and Bacillus abortus (Brucella abortus) was cultivated by Nowak's method as shown on page 3. The two milk samples which yielded Brucella organisms were from eight-months abortion and eleven-months normal birth.

Perhaps the first to report the isolation of Bacterium abortus (Brucella abortus) from milk by direct culture methods was Evans (1915), who described a method by which she isolated and identified Bacterium abortus (Brucella abortus) from milk, using an ordinary lactose agar plate, to which there was added, just before pouring into the plate at a temperature of about 50° C., 10 percent of sterile blood serum. After incubating for four days at 37° C., a certain area of the plate, large enough to include several colonies of Bacterium abortus (Brucella abortus) should they be present, was selected and the colonies were transferred

to nutrient broth containing 1 percent glycerine. The cultures agreed with the description of Bacterium abortus (Brucella abortus) found in the literature.

Up until 1916, no attempts other than isolation by cultural or biological methods had been made in attempting to locate Brucella-abortus-infected udders. However, Coolege (1916) attempted another means of diagnosis by using the agglutination and complement-fixation test as the means of locating infected udders, and in studying the presence of Bacterium abortus (Brucella abortus) in milk. Tests were made using milk and milk serum, instead of the usual method of using blood serum.

From these studies, Cooledge found that there was no apparent connection between the Bacterium abortus (Brucella abortus) antibody-content of the blood and that of the milk. The antibody content of milk may vary from quarter to quarter indicating a source other than the blood stream. His results of agglutination were confirmed by guinea pig inoculation and the direct culture method of Nowak's, using milk sediment.

In another study of the milk in bovine infectious abortion, Giltner, Cooledge, and Huddleson (1916) concluded that milk containing Bacterium abortus (Brucella abortus) antibodies is highly bactericidal for Bacterium abortus (Brucella abortus).

Attempts to improve the method of isolating and recovering the bacillus of cattle abortion through guinea pigs were made by Smillie (1918) who found Bacillus abortus (Brucella abortus) could be readily recovered from the spleen of guinea pigs inoculated

with material containing the bacillus after three to four weeks. This method was useful in recovering the organism from foetal membranes which had come in contact with fecal matter and bedding.

Many investigators up to this time had developed media for growth of Bacillus abortus (Brucella abortus) with some degree of success; however, it was not until Stafseth (1920) developed his liver infusion agar, which was prepared without heating and filtered through glass wool instead of cotton or paper, that there was a marked degree of success in isolation.

Huddleson (1920) in reporting on the isolation of Bacterium abortus (Brucella abortus) from milk by direct cultures stated that one of the most perplexing difficulties usually encountered was the elimination of other faster growing organisms which may be present. He further pointed to four factors which he believed must be considered in making direct culture a comparatively simple process.

1. The medium and its proper preparation.

2. The proper H ion concentration. The H ion concentration lying between pH 6 and 7.6 and the optimum between pH 6.4 and 6.6.

3. The use of an agent which would eliminate fast-growing bacteria.

4. Method of incubation 1:10,000 saturated aqueous solution of gentian violet.

Huddleson cultured organisms from milk sediment obtained from 10 cc of milk centrifuged two hours at 2000 r.p.m. About 0.1 cc of the sediment was drawn from the bottom of the tube by means of a small pipette, then placed on the surface of a solidified gentian-

violet-liver-infusion-agar plate and evenly distributed over a plate by means of a sterile glass rod bent at an angle of 90° C.

The bacteriological examinations were in each instance controlled by inoculating guinea pigs intra-abdominally with 5 cc of whole milk from each quarter and allowing 8 to 10 weeks elapse before autopsying. The spleen and liver were then examined culturally for the presence of Bacterium abortus (Brucella abortus).

The correlation between the plate culture and the guinea pig isolation was very high.

Perhaps the most important work to date was by Huddleson (1921) who, in experimenting with an increased carbon dioxide tension in growing Bacterium abortus (Brucella abortus), found stimulation of growth of the organism not due to a reduced oxygen tension, as formerly believed, but an increased carbon dioxide tension greater than that of the atmosphere. Growth is earlier and more luxuriant.

In a comparative study of whole milk and milk sediment for isolation of Bacterium abortus (Brucella abortus), Fitch and Lubbehusen (1926) concluded that both whole milk and milk sediment are of value in the isolation of Brucella abortus and neither should be used to the exclusion of the other; but, if a quick method is used, the injection of milk sediment is to be preferred.

A study of milk from cows showing no agglutinins for Brucella abortus in their blood serum was made by Carpenter and Parshall (1927). Their studies showed that when a cow has no history of abortion or retained placenta, and her blood serum when diluted

1:60 shows no agglutinins for Brucella abortus, she does not harbor the infection in the udder.

This is in contrast to Kitselman (1927) who found that Bacterium abortus (Brucella abortus) did exist in 3 percent of the cases he tested although the blood tested negative. He also found 12 percent of the cases did not yield Bacterium abortus (Brucella abortus) although the blood tested positive. This is in direct agreement with a conclusion of Cooledge mentioned above.

Additional work of Kitselman agreed with Cooledge in that the blood serum and the milk reaction may vary in the same animal, but his conclusion that guinea pig inoculation yielded a higher percentage of isolation than the direct bacteriological method disagrees with that of Huddleson mentioned above.

Further investigations by Huddleson, Hasley, and Torrey (1927) on the isolation and cultivation of Bacterium abortus (Brucella abortus) revealed that the organism could be measured quantitatively in infected milk with a high degree of accuracy by employing as a culture medium gentian-violet-beef-liver-infusion-agar and incubating in 5 to 10 percent CO_2. Also, that the culturing of the gravity cream layer of milk is just as efficient for determining the presence of Bacterium abortus (Brucella abortus) in milk as guinea pig inoculation, which differs from the observations of Kitselman.

Some important observations on the elimination of Bacterium abortus (Brucella abortus) from cows were made by Gilman (1930), who could not recover Brucella abortus from milk showing agglutination under 1:80, nor from the milk of an animal with a blood

titre lower than 1:320. He did, however, recover Brucella abortus from 53.7 percent of the milk from quarters showing agglutination in dilution of 1:80 or higher. Therefore, he assumed that quarters showing agglutination at 1:80 or above were actively infected with the Bang bacillus and could eliminate the organism at any time. Quarters showing agglutination under 1:80 only in rare instances contained or eliminated the organism.

Further studies on the relation of the milk agglutination titres to the elimation of Bacterium abortus (Brucella abortus) from the udder of the cow were made by Gilman (1931). Brucella abortus were recovered from the milk of 62.9 percent of all the animals showing a positive (1:80) blood titre and 78 percent of those showing a positive 1:80 milk titre in one or more quarters. Gilman concluded that there does seem to be a considerable degree of correlation between the agglutination titre of milk and the presence of Brucella abortus in it.

Work was done by Henry, Traum, and Haring (1932) which added more fuel to the controversial results and methods of the direct culture. In their study of various methods of isolation, they arrived at the conclusion that gravity cream was not as efficient as milk sediment; and, that the direct culture methods were not as effective as guinea pig inoculation.

However, Huddleson (1934), who was an advocate of gravity cream isolation, developed a method for direct culture isolation, which, with a few modifications, is being used extensively today. Huddleson stated that the milk should be collected at or near milking time after the teats were cleaned and the fore milk was

discarded. The milk samples, which consisted of 15 cc of milk in a test tube, were then allowed to set in a cold room for 24 hours, after which the cream was pipetted off and cultured by placing 0.1 to 0.2 cc on the surface of each of two liver-agar plates containing 1 to 200,000 gentian violet. The drops of cream were spread evenly and the plate incubated in 10 percent CO_2 at 37° C. for 72 hours.

Huddleson (1939) modified the above procedure using Difco tryptose agar and a gentian violet dye solution of 1 to 700,000. This modification is still in use today and is generally accepted as the standard method for the isolation of Brucella abortus from milk.

In the undertaken project three methods of isolation were utilized.

1. The standard method explained on pages 10 and 11 under Huddleson 1934 and 1939.

2. The centrifugation-isolation method which used the sediment from centrifuzed milk as the source of inoculum instead of the gravity cream as used by Huddleson in his method of isolation. The centrifugation-isolation method was utilized in the belief that upon centrifuging the white blood cells in the milk would undergo lysis and release phagocytized organisms, thereby resulting in increased isolation.

3. The antigen-addition-isolation method was principally the same as the standard method in that gravity cream was used as the source of inoculum. It differed in that heat-killed Brucella

abortus strain 19 was added to the milk after collecting, and before storing in the refrigerator, with the belief that this heat-killed antigen would tie up the agglutinin; thereby, allowing Brucella organisms to be isolated.

EXPERIMENTAL PROCEDURES

Collection of Milk Samples

Modification one: centrifugation isolation. Samples of milk were collected from the fore and hind quarters of the cow, after the udder and teats were first wiped clean with a cloth that had been rinsed in a chlorine solution. About 10 cc of milk were collected aseptically in screw cap tubes after the first strippings had been discarded. Upon completion of collection, samples were returned to the laboratory to be centrifuged.

Modification two: antigen-addition isolation. Collection of milk samples essentially the same as in modification one except that samples were from each quarter and collected in cotton stoppered tubes. Samples were returned to the laboratory and antigen added before refrigeration.

Standard-method isolation. Same as modification two except no antigen added.

Isolation from Samples

Modification one: centrifugation isolation. Collected

samples were centrifuged at 3000 r.p.m. for thirty minutes, after which the supernatant was pipetted off and the sediment washed in about 20 cc of distilled sterile water. The process of centrifuging and washing was repeated twice, after which one loopful of the sediment was spread uniformly over half of an Albimi agar plate to which had been added 1:700,000 dilution of crystal violet. The second half of the plate was streaked with a loop using the first half as a source of inoculum. The sediment in the specimen tube received 10 ml of Albimi broth and was incubated with the plates at 37° C. in a candle jar containing approximately 10 percent CO_2. After two, five, seven, and ten days, one loopful of the broth was subcultured onto Albimi agar plates as done initially and incubated. The plates were examined after five days and checked for possible Brucella colonies. Examination of colonies consisted of microscopic and macroscopic appearance and agglutination with Brucella sera. Representative colonies giving positive agglutination were inoculated onto tryptose-agar slants for identification of Brucella species. These tryptose-agar slants were incubated at 37° C. under 10 percent CO_2 for two days, at the end of which time they were examined and checked for Brucella by agglutination with positive Brucella sera made from inoculating rabbits with Brucella abortus strain 19.

The agglutination method was as follows: a suspension of organisms and saline was placed on a glass plate. This suspension was made by mixing several loopfuls of sterile saline and a loopful of organisms taken from the agar slant. Several loopfuls of

serum were added to the suspension and then the plate was heated gently over a flame. Agglutination was shown by clumping of cells.

The Albimi brucella agar was made up as follows: to the one liter of Albimi brucella broth, which contains the following ingredients:

Peptone M	20.0 grams
Dextrose C.P.	1.0 grams
Yeast antolysate	2.0 grams
Sodium chloride	5.0 grams
Sodium bisulfate	0.1 grams

pH 7.0 ±

was added 2 percent, or 20 grams of agar-agar.

The Albimi broth media was prepared by adding 28 grams of the commercially prepared mixture to one liter of distilled water. The sterilization was at fifteen pounds pressure (121° C.) for twenty minutes.

Modification two: antigen addition isolation. One sample from each quarter of each cow was mixed thoroughly with 1 ml of the Brucella abortus strain 19 antigen by tilting tubes back and forth. Samples then were allowed to stand in refrigerator overnight or approximately 24 hours, by which time the cream would be on the surface. One-tenth ml of cream from each sample of each quarter was inoculated onto tryptose-agar plates containing crystal violet in a final concentration of 1 to 700,000. This amount of dye is sufficient to suppress practically all gram-positive organisms which might otherwise interfere. To secure the proper concentration, .14 cc of a 0.1 percent solution of crystal violet was added to 100 cc of tryptose agar after cooling, but prior to pouring the plates. The 0.1 ml of cream was smeared

over entire plate by means of a sterile wire with a right-angle bend. Plates were labeled according to cow, quarter, and presence or absence of Brucella abortus strain 19 antigen.

The composition of the tryptose agar was as follows: Bacto tryptose - 20 grams; Bacto dextrose - 1 gram; sodium chloride - 5 grams; and Bacto agar - 15 grams in one liter of water. pH 6.8 ±.

The inoculated plates were sealed under 10 percent carbon dioxide tension, using the candle-jar method, and incubated at 37° C. for five days. At the end of this period, the plates were examined for Brucella colonies which generally appear as purplish, smooth, transparent, hemispherical colonies, about 1 to 5 mm in diameter. Occasionally the colony characteristics vary from smooth to rough, and intermediate stages; but for the most part were found in the smooth stage.

Colonies examination were both macroscopically and microscopically as done under modification one and representative colonies giving positive agglutination were processed in the same manner as described on pages 12, 13 and 14 under modification one.

Standard-method isolation. Essentially the same as modification two, except that no antigen was added to milk samples.

Identification of the Organism from Samples

Modification one: centrifugation isolation. The identification of the Brucella types was checked by their behavior in the

presence of bacteriostatic dyes; namely, thionin 1:200,000 and basic fuchsin 1:100,000. Brucella melitensis and Brucella suis will grow on tryptose agar containing thionin, while Brucella abortus is inhibited. Brucella melitensis and Brucella abortus develop on tryptose agar containing basic fuchsin while Brucella suis is inhibited.

Modification two: antigen-addition isolation. The procedure is the same as modification one.

Standard-method isolation. The procedure is the same as modification one.

Preparation of Antigen

Modification two: antigen addition. Cultures of smooth type Brucella abortus, strain 19 obtained from Professor V. D. Foltz, were grown on tryptose-agar slants. These cultures were checked for purity and then to each culture tube there was added 5 cc of sterile physiological saline solution to remove and suspend the bacteria. One cc of the suspension of organisms was used for seeding Blake bottles containing 100 cc of potato dextrose medium. The medium was composed of 5 grams sodium chloride, 10 grams bacto peptone, 5 grams beef extract, 10 grams dextrose, 30 grams washed agar, and the filtrate from 250 grams of raw potatoes made up to a liter. The seeded Blake bottles were incubated at 37° C. for 72 hours. At the end of the incubation period, the Blake bottles were removed from the incubator and

examined for purity. To those bottles that showed no contamination were added aseptically 10 cc of physiological saline solution. The bottles were rotated until all the cells were free of the medium and in suspension. Then the liquid contents were filtered through sterile guaze into sterile 6-ounce bottles. The suspension of organisms were killed with heat by steaming for fifteen minutes and the absence of living organisms was checked by inoculating tryptose-agar slants. The suspensions were then transferred aseptically into screw-cap tubes and centrifuged for fifteen minutes at 3500 r.p.m. in a Servall angle centrifuge. The supernatant from the tubes was removed with a sterile pipette and sterile saline added and the cells resuspended. The cells again were centrifuged for the same time and speed as before the supernatant removed, and sterile saline added to resuspend the cells up to the original volume.

The cell suspensions were standardized by the Macfarland Nephelometer method to a concentration of 180 times nephelometer tube number 1. The pH of the suspension was adjusted to 6.8 by the electrometric method.

RESULTS

Modification one: centrifugation isolation. Sixteen samples from five cows that had at one time been shedding Brucella organisms were cultured by modification one. Numerous colonies of organisms appeared on the plate, but for the most part

Table 1. Isolation of *Brucella abortus* from milk by Modification One.

Cow no.	Date of collection	Date of isolation	Quarters Hind	Quarters Front	Isolation Orig., 2, 5, 7, 10
391A	5/8/51	5/14/51	Hind		Original culture
391A	6/9/51	———	———		———
391A	6/16/51	———	———		———
392A	5/9/51	———	———		———
388A	5/9/51	———	———		———
388A	6/16/51	———	———		———
372A	6/9/51	———	———		———
128A	6/9/51	6/17/51	Hind		2-day subculture

Table 2. Isolation of *Brucella abortus* from milk by Modification Two.

Cow no.	Date of collection	Date of isolation	Standard method isolation: Quarter samples # 1	2	3	4	Antigen addition isolation: Quarter samples # 1	2	3	4
391A	3/2/51	3/10/51	#	-	-	-	#	-	-	-
391A	3/15/51	3/23/51	#	-	-	-	#	-	-	-
391A	4/13/51	4/24/51	#	-	-	-	#	-	#	-
388A	3/2/51	-------	-	-	-	-	-	-	-	-
388A	3/15/51	-------	-	-	-	-	-	-	-	-
372A	3/2/51	-------	-	-	-	-	-	-	-	-
219A	3/2/51	-------	-	-	-	-	-	-	-	-
392A	3/15/51	-------	-	-	-	-	-	-	-	-
392A	4/13/51	-------	-	-	-	-	-	-	-	-
450A	3/15/51	3/23/51	-	-	#	-	#	-	#	-
450A	4/13/51	4/24/51	-	-	#	-	#	-	#	-
128A	4/13/51	4/24/51	#	#	#	-	-	#	#	-

Quarter 1 = right hind
Quarter 2 = right front
Quarter 3 = left hind
Quarter 4 = left front
Isolation occurred
- Isolation (none)

Table 3. Total count of Brucella abortus by standard and Modification Two Method.

Cow no.	Date of collection	Date of isolation	Standard method Quarter sample total count 1	2	3	4	Antigen addition method Quarter sample total count 1	2	3	4
394A	7/2/51	-------	-	-	-	-	-	-	-	-
327A	7/2/51	-------	-	-	-	-	-	-	-	-
255A	7/2/51	-------	-	-	-	-	-	-	-	-
128A	7/2/51	7/9/51	2	21	187	0	8	11	26	0
128A	7/3/51	7/10/51	0	14	9	0	0	28	55	0
128A	7/9/51	7/17/51	16	283	360	0	61	268	86	0
128A	7/18/51	7/26/51	0	0	0	0	0	135	0	0
128A	7/23/51	7/30/51	1	34	40	0	2	86	200	0
128A	7/25/51	8/2/51	1	62	21	0	14	178	52	0
391A	7/3/51	7/10/51	4	0	0	0	8	0	11	0
391A	7/9/51	7/17/51	0	0	0	0	23	0	0	0
391A	7/18/51	7/26/51	0	0	0	0	10	0	0	0
391A	7/23/51	7/30/51	4	0	0	0	12	0	0	0
391A	7/25/51	8/2/51	10	0	0	0	16	0	0	0

Quarter 1 = right hind Quarter 3 = left hind
Quarter 2 = right front Quarter 4 = left front
Values recorded represent colony numbers in 0.1 ml of milk

Table 4. Vaccination, *Brucella* titer, and isolation of experimental animals.

Cow no.	Date of vaccination	Date of revaccination	Titers	and dates of titers	Isolation dates
372A	1/20/49	-------	1:400	1/20/49	-------
			1:400	12/6/48	
			1:400	12/16/48	
			1:400	2/5/49	
			1:400	2/11/49	
			1:400	2/18/49	
			1:400	11/29/49	
128A	5/10/44	7/27/44	1:400	12/6/48	1/15/49
		1/20/49	1:400	12/16/48	4/24/51
			1:1600	1/20/49	6/9/51
			1:1600	2/2/49	7/2/51
			1:2400	2/11/49	7/10/51
			1:2400	2/18/49	7/19/51
					7/20/51
					7/23/51
					7/25/51

Table 4. (cont.)

Cow no.	Date of vaccination	Date of revaccination	Titers and dates of titers		Isolation dates
388A	5/5/48	-------	1:500	4/1/48	-------
			1:500	4/13/48	
			1:500	5/5/48	
			1:500	5/14/48	
			1:500	5/21/48	
			1:500	5/31/48	
391A	11/3/45	10/1/48	1:3000	9/10/48	3/10/51
			1:2400	9/24/51	3/23/51
			1:2400	10/1/48	4/24/51
			1:2400	10/9/51	5/14/51
			1:2400	10/14/48	7/10/51
			1:1600	10/26/48	7/17/51
			positive	11/29/49	7/30/51
					8/2/51

Table 4. (cont.)

Cow no.	Date of vaccination	Date of revaccination	Titers and dates of titers		Isolation dates
392A	1/20/49	-------	1:1600	12/6/48	-------
			1:1600	12/16/48	
			1:1600	1/2/49	
			1:1600	2/2/49	
			1:1600	2/11/49	
			1:1600	2/18/49	
			positive	12/2/49	
394A	1/9/46	-------	1:200I	11/3/50	-------
450A	5/5/48	-------	1:400	4/1/48	3/23/51
			1:400	4/16/48	4/24/51
			1:400	5/5/48	-------
			1:400	5/14/48	
			1:400	5/21/48	
			1:400	5/31/48	
			1:200	2/16/48	

Table 4. (cont.)

Cow no.	Date vaccination	Date of revaccination	Titers and dates of titers		Isolation dates
219A	5/5/48	-------	1:400	4/1/48	-------
			1:400	4/16/48	
			1:400	5/5/48	
			1:400	5/14/48	
			1:400	5/21/48	
			1:400	5/31/48	
			negative	12/2/49	
255A	10/1/48	-------	0	9/10/48	-------
			0	9/25/48	
			0	10/1/48	
			1:50?	10/9/48	
			1:800	10/16/48	
			1:800	10/26/48	
			1:200p	5/9/48	
			1:50s	4/4/51	

Table 4. (concl.)

Cow no.	Date of vaccination	Date of revaccination	Titers and dates of titers		Isolation dates
327A	10/1/48	-------	1:50	9/10/48	-------
			1:50	9/27/48	
			1:50	10/1/48	
			1:400	10/9/48	
			1:400	10/16/48	
			1:400	10/26/48	
			1:50	5/9/49	
			1:100I	4/14/51	

macroscopic, microscopic, and agglutination tests proved they were not Brucella organisms. Of the 80 plates prepared from the sixteen samples (original cultures and 2, 5, 7, 10-day subcultures) only two plates showed Brucella organisms and in these only single colonies found on each plate.

The isolations identified as Brucella abortus were from the hind quarters of cows 128A and 391A, as shown in Table 1. The isolations were from the original culture in the sample from 391A and the two-day subculture in the sample from 128A.

Modification two as compared to standard-method isolation. Ninety-six samples from seven cows that had at one time been shedding Brucella organisms were employed in the first experiment, using modification two and standard-method isolation. Several to numerous Brucella colonies were isolated by both the standard method and modification-two method. All organisms isolated were identified by agglutination tests and differential dye media as Brucella abortus. In no animal did isolation occur by either standard or modification method and not by the other. However, isolation by quarter samples did show some difference in results as shown by Table 2.

Three of the seven animals and eighteen of the ninety-six samples tested yielded the Brucella abortus organisms.

Data obtained in experiment two, using the same methods are recorded in Table 3 in terms of the total number of isolated Brucella abortus colonies by the two methods. In this experiment five cows were used, but only two extensively. Since these two

gave repeated isolations, it was possible to get a better comparison of total counts by standard and antigen-addition method. In all, 112 samples of milk were taken from these five cows in these experiments. Of the 112 samples, thirty-eight yielded Brucella organisms. These were from cows 391A and 128A. Table 3 shows a comparison of isolation by the two methods.

The total number counts reveal that the addition of a Brucella antigen to the milk gave rise to more colonies and in three cases resulted in isolations where the standard method gave no isolations.

DISCUSSION

As is evident from the data in Tables 1, 2, and 3, that isolation of Brucella abortus by the three methods, from the supposedly Brucella shedding cows, has been extremely variable in that very few of the total number of milk samples tested from these reactors gave positive Brucella abortus colonies. This lack of consistent isolation might be traced back to the original supposition that Brucella may be shed at one time and not at another, intermittently so-to-speak. If this was the case, then the results of isolation were negative in so far as differences in isolation by standard versus modified procedures is concerned. However, wherever isolation from an animal occurred, it occurred not intermittently but on a series of successive dates covering up to five consecutive isolations, suggesting that perhaps some factor besides procedures was operating.

It should be noted that in this experiment all animals were from the Kansas State College Dairy Herd; and thus, the experiment could not be controlled in that the animals were available only when on the milk line. In some cases the animals were removed from the line (milk) for various reasons; this accounted for the examination of specimens from these animals only once or twice. Also, another factor which no doubt interferred with the results was the fact that all these animals had been vaccinated as calves and some had revaccinations throughout their history. It is entirely possible that the vaccination program influenced the lack of isolation from some of the reactors. Table 4 shows history of cows used in the experiment.

Some very interesting work has been done on this problem of vaccination. In this program a method has been devised whereby it is impossible to tell by the blood agglutination test if an animal's titer is an infection titer or a vaccination titer. Various investigators have attempted to differentiate between the two titers. The methods used are the whey agglutination, Brucella ring test, and the anamnestic blood reaction.

Important work on the anamnestic reaction was done by Dick, Venzke, and York (1947) who believed that any animal that was not stimulated to the production of agglutinin by the intra-muscular injection of 5 cc of strain 19 vaccine within a maximum of fifteeen to seventeen days was an infected animal.

Venzke (1948) in a follow-up report stated that in an infected animal, the Brucella organisms were continually present as a

stimulus to the antibody-producing mechanism. With such a continual stimulation, the antibody-producing mechanism possibly becomes refractory and fails to respond to an antigenic stimulus.

This work was supported by Barner (1949) whose results paralleled those of Dick, Venzke, and York.

The answer to the intermittent isolation problem may be simply that if isolation does not occur, the animal is not infected. However, other factors such as differentiation of titers, and other methods of isolation, must be considered and experiments worked out to determine their possible influence.

The isolation of Brucella organisms by the centrifugation method was based upon the supposition that by the throwing down of the white blood cells in the milk samples lysis would occur and result in the release of the phagocytized organisms.

As Table 1 shows, success for the most part was lacking except in two isolated cases. This method was not followed extensively because of the following reasons:

1. Poor isolation.
2. Excessive handling of samples in centrifugation, leading to contamination.
3. Frequent subculturing.
4. Excessive amounts of material needed.
5. Extremely undesirable odors.
6. Too much time needed in isolation.

In noting the results of isolation by the antigen-addition method in comparison with the standard method as described by Huddleson, seventeen instances occurred where isolation from a

udder was accomplished by antigen addition, while only fourteen isolations were accomplished by standard method. The number of colonies varied in the different methods of isolation, and usually were greater from the antigen-addition method. However, there were a few exceptions to this. It was also noted that in samples from individual quarters, isolation did occur more often using antigen than it did by standard methods.

Of the quarters sampled, only the left front completely failed to yield organisms on culture plate. It would seem necessary then in efforts to detect infection to emphasize the examination of all four quarters. Cooledge (1916) believed the vaginal discharge on the tail, and consequent spreading by switching resulted in greater isolation from the rear quarters.

The theory behind the addition of antigen to the sample was to tie up the agglutinins, thereby allowing Brucella organisms to be isolated. Some of the data tend to indicate better isolation by antigen addition. With a controlled experimental program this method might have merit, since it has been shown to be as good if not a better method of isolation than the standard methods.

SUMMARY

1. In isolation by centrifugation, using sixteen samples from five positive Brucella reacting cows and making eighty plate cultures, only two plates showed organisms which were identified as Brucella abortus.

2. Isolations using the antigen-addition method resulted in ten isolations of Brucella abortus from three cows using forty-eight samples.

3. Standard-method isolation yielded eight isolations of Brucella abortus from forty-six samples.

4. Using a total count and antigen addition resulted in twenty-one isolations from five cows using 56 samples.

5. A total count and standard-method isolation resulted in seventeen isolations from five cows using 56 samples.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Professor V. D. Foltz for his helpful guidance during this problem. Grateful thanks are extended, also, to Dr. P. L. Gainey for his assistance in the preparation of this manuscript.

BIBLIOGRAPHY

Bang, B.
Infectious abortion in cattle. Jour. Compar. Path. and Ther. 10:125-149. 1897.

Barner, R.
The anamnestic reaction as an aid in the differentiation of vaccinal from infection titers of Brucella abortus in cattle. Unpublished report. 1949.

Carpenter, C. M., and C. J. Parshall.
A study of milk from cows showing no agglutinins for Brucella abortus in their blood streams. Cornell Vet. 17:234. 1927.

Cooledge, L. H.
A study of the presence of Bacterium abortus (Bang) in milk. Mich. Agr. Expt. Sta. Tech. Bul 33. October, 1916.

Dick, J. R., W. G. Venzke, and C. York.
A method for differentiating between vaccinal titers and infection titers of Brucella abortus in cattle. Amer. Vet. Med. Assoc. Jour. 111:255-258. 1947.

Evans, A. C.
Bacillus abortus in market milk. Wash. Acad. Sci. Jour. 5:122-123. 1915.

Fabyan, M.
A note on the presence of Bacillus abortus in cows milk. Jour. Med. Res. Volume 28. May, 1913.

Fitch, C. P., and R. E. Lubbehusen.
A study of whole milk and milk sediment for the isolation of Bacterium abortus (Bang). Cornell Vet. 16:46-53. 1926.

Giltner, W., L. H. Cooledge, and I. F. Huddleson.
A study of the milk in bovine infectious abortion. Amer. Vet. Med. Assoc. Jour. 50:156-167. November, 1916.

Gilman, H. L.
The elimination of Bacterium abortus in the milk of cows. Cornell Vet. 20:106-119. 1930.

Gilman, H. L.
Further studies on the relation of the milk agglutination titers to the elimination of Bacterium abortus from the udder of the cow. Cornell Vet. 21:243-249. 1931.

Henry, B. S., J. Traum, and C. M. Haring.
Methods for the isolation of Brucella abortus. Hilgardia, A Journal of Agricultural Science, California Agr. Expt. Sta. 6:356-377. 1932.

Huddleson, I. F.
Isolation of Bacterium abortus from milk. Michigan Agr. Expt. Sta. Tech. Bul. 49. 1920.

Huddleson, I. F.
The importance of an increased carbon dioxide tension in growing Bacterium abortus (Bang). Cornell Vet. 11:210-214. 1921.

Huddleson, I. F.
Brucellosis in man and animals. The Commonwealth Fund. 1934.

Huddleson, I. F.
Brucellosis in man and animals. The Commonwealth Fund. 1939.

Huddleson, I. F., D. E. Hasley, and J. P. Torrey.
Further studies on the isolation and cultivation of Bacterium abortus. Jour. Infect. Dis. 40:352. 1927.

Kitselman, C. H.
Cattle abortion disease investigation. Unpublished Master's Thesis. Kansas State College. Manhattan, Kansas. 1927.

McNeal, W. J., and J. E. Kerr.
Bacillus abortus of Bang, the cause of contagious abortion in cattle. Jour. Infect. Dis. 7:469. 1910.

Melvin, A. D.
Infectious abortion in cattle and the occurrence of its bacterium in milk. Introductory Statement, 28th Annual Report, Bureau of Animal Industry. 137-138. 1911.

Mohler, J. D., and J. Traum.
Infectious abortion of cattle. 28th Annual Report, Bureau of Animal Industry. 1911.

Nowak, J.
Le bacille de Bang et sa biologie. Annales Institute Pastuer. 22:541-556. 1908.

Schroeder, E. C., and W. E. Cotton.
The bacillus of infectious abortion found in milk. 28th Annual Report, Bureau of Animal Industry. 1911.

Smillie, E. W.
An improvement in the method of isolating and recovering the bacillus of cattle abortion through guinea pigs. Jour. Expt. Med. 28:585-605. 1918.

Smith, T., and M. Fabyan.
The pathagenicity of Bacillus abortus (Bang). Centralblatt fur Bakteriologie (abt.1). 61:549. 1912.

Stafseth, H. J.
A few notes on the isolation and cultivation of Bacterium abortus with special reference to liver and spleen media. Mich. Agr. Expt. Sta. Tech. Bul. 49. II 1920.

Venzke, W. G.
The differentiation of cattle with Brucella abortus infection titers and cattle vaccination with Brucella abortus strain 19. North American Veterinarian. 29:484-488. 1948.

RELATIVE MERITS OF STANDARD VERSUS MODIFIED PROCEDURES FOR ISOLATION OF *BRUCELLA ABORTUS* FROM MILK

by

DEANE FAY WEBER

B. S., Jamestown College, 1950

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1952

The isolation of Brucella abortus from milk is one of the specific means of detecting Brucella infection, upon which depends the answer to many questions being asked concerning brucellosis. Yet, due to the complexity of the nature of Brucella abortus, the problem of isolation still presents some difficulties. One important unsolved isolation problem is the frequently reported intermittent isolation of Brucella abortus from cows showing a positive Brucella blood agglutination titer.

This intermittent isolation of Brucella abortus from the milk of positive Brucella reactors suggests the possibility of a lack of accuracy or ineffectiveness in the present methods of isolation. Therefore, it would be decidedly advantageous if an accurate and delicate method of isolating Brucella abortus from milk were available. One could then be reasonably certain of the presence or absence of these organisms in milk depending upon his findings. It was with the possibility in mind of improving the delicacy and accuracy of present day methods of isolating Brucella from milk that this project was undertaken.

In the study three methods were utilized.

1. The standard method which utilized the gravity cream as a source inoculum. In this method 15 cc of milk were collected aseptically in test tubes after the fore milk was discarded. The milk samples were then allowed to set in a cold room for 24 hours, after which the cream was pipetted off and cultured by placing 0.1 to 0.2 cc on the surface of Difco tryptose agar plates containing 1 to 700,000 gentian violet. The crops of cream were spread

evenly and the plates incubated in 10 percent CO_2 at 37° C., for 72 hours.

2. The centrifugation-isolation method which used the sediment from centrifuged milk as the source of inoculum instead of the gravity cream as used above. The centrifugation-isolation method was utilized in the belief that upon centrifuging the white blood cells in the milk would undergo lysis and release phagocytized organisms; thereby, resulting in increased isolation.

3. The antigen-addition-isolation method was principally the same as the standard method in that gravity cream was used as the source of inoculum, but differed in that heat-killed Brucella abortus strain 19 was added to the milk after collecting and before storing in the refrigerator with the belief that this heat-killed antigen would tie up the agglutinin, thereby, allowing Brucella organisms to be more readily isolated.

Using centrifugation isolation, 16 samples from cows that had at one time been shedding Brucella organisms were cultured. Of the 80 plates prepared from the 16 samples only two plates showed Brucella organisms and in these only single colonies were found on each plate. Factors such as poor results, excessive materials, excessive time spent, and excessive handling forced the abandonment of this particular phase.

Comparing antigen-addition-isolation with standard isolation methods 96 samples from 7 cows that had at one time been shedding Brucella organisms were employed in the first experiment using these two methods. In no animal did isolation occur by either

method and not by the other. However, isolation from quarter samples did show some differences. Isolations using the antigen-addition method resulted in 10 quarter isolations, whereas, standard method isolation yielded only 8 quarter isolations.

In the second experiment using a comparison of antigen-addition isolation with standard isolation method the total number of isolated Brucella abortus colonies were counted. The total number counts reveal that the addition of a Brucella antigen to the milk gave rise to more colonies and in three cases resulted in isolations where the standard gave no isolations. The antigen-addition-isolation resulted in 21 quarter isolations while the standard resulted in 17 quarter isolations.

Results from this project tend to support the method of antigen-addition in isolating Brucella organisms from infected udders since more colonies and isolations were found by this method than by the others. With a controlled experimental program this method would have considerable merit.