Comparison of liquid and solid selective media for isolation of *Brucella abortus* from vaginal secretions of cattle

by

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INTRODUCTION

Brucella abortus (B. abortus) is a causative agent of contagious abortion in cattle, also infecting other domestic animals and man.

Bovine brucellosis causes a significant economic loss to the U.S. livestock producers; often the only visible evidence is the loss of calf crops. However, reductions in fertility and milk production are important. Brucellosis is also of public health concern since the disease can be transmitted from animals to man.

The shedding of Brucella through the genital discharges at the time of abortion is considered the most significant way of transmitting the disease from infected to non-infected cattle, although shedding also occurs in milk and other secretions.

The detection of infected animals by routine serological tests has been widely used; however, these methods have limited accuracy. Therefore, the recovery of Brucella by bacteriological techniques is of great importance for a definitive diagnosis of the disease. Important problems which influence the cultural isolation of B. abortus may include: overgrowth by non-brucella organisms when culturing contaminated specimens, detecting minimal numbers of organisms present in carrier animals, and the interference of isolation by the presence of agglutinins and other antimicrobial inhibitors in the tissues and body fluids. Thus, the development of practical, reliable diagnostic methods is still needed. Improved selective media could increase efficiency of the isolation of brucellae from contaminated sources and sources containing minimal numbers. This

would be of value for: 1) detection of actively infected animals, 2) identification of carrier animals in problem herds, and 3) brucellosis research.

The objectives of this research were to: 1) develop a selective liquid medium useful for the isolation of B. abortus from sources in which only a few organisms are present, while preventing overgrowth by non-brucella organisms, 2) to determine the shedding pattern of B. abortus in vaccinated and challenged cattle by the use of the selective enrichment broth, and 3) to compare the use of the selective enrichment broth technique to the routine direct streak plate method using solid media for culturing B. abortus from the vaginal secretions of cattle.

This thesis consists of two separate, but related parts:

1) preliminary studies of the development of a selective enrichment broth,
2) the comparison of the enrichment broth and direct streak plate methods used to determine shedding patterns of B. abortus in the vaginal secretions of adult cattle.

LITERATURE REVIEW

History

Brucellosis is an infectious disease primarily of domesticated animals and is transmissible to man. The cause of the disease was established by Bruce in 1877, by the isolation of the bacterium, which he named Micrococcus melitensis, from the spleens of patients dying of Malta fever (66). Zammit in 1905, found that goats on the Isle of Malta were infected and served as a reservoir in transmitting the disease to man through milk products. In 1897, Bang isolated B. abortus from the abortion products of cows and it was shown to cause infectious abortion in cattle (3). It was not until 1918, through research conducted by Evans, that the organisms described by Bruce and Bang were closely related and as a result the genus Brucella was established (1).

The genus Brucella presently contains six species:

1) Brucella melitensis (B. melitensis) causes brucellosis in sheep and goats, and a most important species to cause disease in man
2) Brucella abortus (B. abortus) causes contagious abortion in cattle, and frequently infectious to man
3) Brucella suis (B. suis) mainly pathogenic for pigs, although certain biotypes infect rabbits, reindeer, and man
4) Brucella ovis (B. ovis) causes ram epididymitis
5) Brucella canis (B. canis) infectious to dogs
6) Brucella neotomae (B. neotomae) isolated from desert wood rat.

Characteristics of the genus Brucella

Members of the genus Brucella are non-motile, short, non-spore-forming gram-negative coccobacilli. Colonies are small, round, convex, smooth, and translucent. Growth is relatively slow, especially on initial cultivation. Certain strains and biotypes are CO_2 dependent, but others may grow in aerobic conditions (1, 57, 66). Brucellae show little fermentative action on carbohydrates. Their nutritional requirements are relatively complex, and a variety of amino acids and vitamins has been found to be essential for growth (35, 69). Therefore, special media are often required.

Mode of Transmission

Bovine brucellosis has been reported as a worldwide problem (103). Eradication programs in the United States for bovine brucellosis which are based on vaccination of calves and the elimination of infected animals have not been completely successful (15).

Information pertaining to interherd spread is limited. Transmission of the disease has been shown to occur between dam and offspring, although the major route of transmission arises from infected uterine discharges of the infected cow contacting another (56).

Brucellosis in man caused by B. abortus is of animal origin contracted by the handling of diseased animals and their products, or ingestion of infected meat or milk (66, 70). Therefore, the prevention of brucellosis in man is dependent on its control in animals.

Diagnosis of Bovine Brucellosis

Many procedures have been used to diagnose bovine brucellosis including: direct bacteriological isolation, serological methods, animal inoculation, and skin tests (1, 44, 109). Alton et al. (1) stated that "incontrovertible evidence of Brucella infection is obtained by the isolation and identification of the organism. Since it is not always possible to isolate the causal organisms from infected patients, serological tests play a major role in the routine diagnosis of brucellosis." Bacteriological isolations of brucella may be made from unpasteurized dairy products from infected animals and from specimens such as blood, tissues, placenta, aborted fetus, and vaginal mucus (57, 70).

A number of serological tests based on the presence of brucella antibodies in the serum, milk, vaginal mucus, and seminal plasma of infected animals have been used (1). All serological tests used at present have certain limitations which are in part compensated by the employment of a battery of tests. There are no reports that indicate any serological test is capable of accurately distinguishing between antibody titers resulting from vaccination and those due to field strain infection. Also serological tests may not detect localized infections. For example, B. abortus has been isolated from the semen of bulls in which a negative serum agglutination test was reported (56). However, the agglutinin titer of semen plasma is often higher than in serum which may be detected, thus indicating infection.

Animal inoculation is not routinely used because it has a serious disadvantage in that the results may not be available until six weeks after

the sample has been collected. Isolation of brucellae from guinea pig lesions along with rising agglutination titers have proved to be a reliable diagnostic procedure (70). When highly selective media are used, isolation rates equal to, if not greater than those of guinea pig inoculation may be achieved (82).

Diagnosis of brucellosis in cattle by skin tests has not been successful since the test appears to lack specificity and sensitivity (44).

Because of these limitations, diagnosis of bovine brucellosis has for the most part occurred in the laboratory using cultural methods and serological tests.

Immune Response to B. abortus in the Bovine Genital Tract

Infection by B. abortus stimulates both cellular and humoral immune systems of the host defenses. The pathogenicity of brucella is mainly related to the organisms ability to survive and multiply within the host phagocytes (97, 111). The protective function of the immunoglobulins in bovine secretions is poorly understood (12, 13). Serum agglutinins stimulated by vaccination with B. abortus strain 19 have been shown to be of IgG, IgM, and IgA classes (79, 84).

Brucella has been shown to produce cervicovaginal agglutinins which appear prior to or in the absence of serum antibody (80). Cervicovaginal agglutinins stimulated after vaccination have not been identified. The possibility of local synthesis of IgG in the reproductive tract of actively immunized animals is not excluded. Various investigators have characterized and quantitated the natural immunoglobulins of cattle

demonstrating local synthesis or selective transport of all secretory IgA, as well as a portion of IgG and IgM in vaginal secretions (13, 19, 22, 86, 87, 114). Serum components derived by transudation accounted for a much higher proportion of total protein in vaginal mucus than in other external secretions. The mechanism of antibody immunity at the mucus membrane surface remains unclear as does the role of serum-derived and locally synthesized immunoglobulins in resistance (16, 22, 62). Complement-dependent opsonization plays an important role in specific and non-specific phagocytosis of brucella in the blood (85, 111). This may also be a possible mechanism by which immunity is mediated in the vaginal secretions because of the high proportion of serum proteins derived by transudation (16, 22, 62).

Shedding of B. abortus in the Vaginal Secretions of Cattle

Bovine brucellosis is characterized as a generalized infection, but abortion is the most frequently observed clinical sign which is only pertinent if the infected animal is pregnant. Most infected animals abort only once, although brucella may be shed during subsequent parturitions (40, 64). The incubation period is variable from a few days to several weeks determined by the stage of gestation. Incubation periods are shorter when exposure takes place late in gestation and reduced exposure doses lengthens incubation and decreases the chances of abortion (64, 80). Schroeder stated that B. abortus has a favorite habitat in cattle which was found to be the udder, the uterus during gestation and shortly after abortion and parturition, and lymph nodes associated with these

organs (95). Erythritol was demonstrated in bovine allantoic, amniotic fluids (81, 98, 99), the chorion, and cotyledons (116). The growth-promoting activity of maternal and fetal tissue extracts was found to be directly related to the erythritol content (49). Basic and characteristic tissue changes can occur by the interaction of the mononuclear phagocytes and lead to the formation of granulomas (8, 68). Lesions are sometimes associated with pneumonia and enteritis of newborn calves.

Many investigators have studied the genital shedding of B. abortus in pregnant and non-pregnant cattle (3, 8, 17, 18, 33, 34, 64, 82, 83, 95). In 1897, Bang first reported that a large number of organisms were shed at the time of abortion in vaginal discharges and also in the placenta (3). Most early reports dealt with the finding of B. abortus in genital organs at autopsy rather than in relation to the period of elimination of the organisms by way of the vagina (17, 95). Cotton stated that at autopsy, the maximum length of time B. abortus were found in the uterus after abortion was 52 days (17).

It is generally accepted that the excretion of B. abortus through the vaginal discharges of cattle after normal or abnormal parturitions have been reported to be massive and continuous for two weeks. Shedding of B. abortus has then been shown to diminish by four to five weeks, after which it has been shown to be intermittent in 5-10% of the cows for up to two years (8, 34, 64).

In non-pregnant cows and before parturition, the occurrence of B. abortus in the vaginal discharge was reported as slight and discontinuous.

Only a few cases have been reported of the isolation of B. abortus from vaginal discharges of cows before parturition (33, 64, 83). The appearance of B. abortus in the vagina is believed to be directly related to the freeing of the cervical plug, which allows the release of the uterine discharges (83). Therefore, questions have been raised as to whether false-negative results have been due to inadequate methods of isolation or perhaps false isolations have been due to contamination of vaginal samples by urine or feces containing Brucella (33, 83).

Characterization of Strain 19

Vaccination with the attenuated B. abortus strain 19 has been used for many years for the protection of cattle against brucellosis. Strain 19 replaced virulent B. abortus for vaccination because it was observed that when lactating cows were vaccinated, the organism frequently became established in the udder resulting in carriers and shedders (18). Strain 19 did not become localized in the udder except in rare cases when high doses were injected into pregnant cows which produced abortion and strain 19 was isolated from the genital tract (8). This problem was found to be eliminated with calfhood vaccination of animals at 4 to 8 months of age producing a resistance that was comparable to animals vaccinated as two-year-olds. Calfhood vaccination also reduced the problem of persistent post-vaccinal titers in adult animals (50).

Adult vaccination with strain 19 is again being used in problem herds infected with brucella. B. abortus isolates are occasionally recovered from adult cattle so vaccinated. These isolates must be distinguished

from field strains of B. abortus biotype 1.

Strain 19 does not require added carbon dioxide for growth as do most field strains. Characterization studies have indicated that strain 19 differs from other carbon dioxide-independent field strains of B. abortus in that they do not grow on a medium containing thionin blue (1:500,000) or penicillin (5 u/ml) (11, 46). The ability of strain 19 cultures to grow on a medium containing erythritol (1 mg/ml) and their ability to oxidize erythritol is variable (11, 46, 71). The inability of resting cells to oxidize erythritol is a characteristic unique to the USDA strain 19. The factors responsible for the variation in erythritol sensitivity of strain 19 cultures are not known (11).

Differential characteristics used in routine identification tests to distinguish strain 19 from other B. abortus biotypes are listed in Table A1. Quantitative differences have been shown between strain 19 and other strains of B. abortus in the oxidative rates of certain substances including D-alanine, L-glutamic acid, d(+)-galactose, and D-ribose (11).

Development of Basal Media

Since the first isolation of B. melitensis by Sir David Bruce in 1877, on a peptone-beef infusion agar medium, a vast number of media has been developed for the isolation of brucella (89). Through the years, with a better understanding of the nutritional requirements and the awareness of certain substances toxic to brucella, many of the difficulties originally experienced in isolation and cultivation have been overcome (35). Reports indicate that certain strains of B. abortus are more

difficult to cultivate initially than others due to additional nutritional requirements and increased CO_2 atmosphere (45, 117).

Brucella abortus was first isolated on semi-solid gelatin agar containing 33% serum, from the abortion products of cows, in 1897 by Bang (3). Schroeder and Cotton isolated B. abortus on agar containing 6% glycerine and 5% ox-bile (95).

Holth was first to describe the use of spleen and liver infusion media. These proved superior to the wide variety of media containing extracts of bovine uterine wall, fetal membranes and fetuses, amniotic fluid, blood clot, and glycerine for supporting the growth of B. abortus (101).

Commercial peptones and meat infusion broths were developed between 1912-1920 using preparations from pig or bovine liver (102). However, because of variation in liver tissue, meat extracts were replaced by a peptone prepared from pancreatic digest of casein (40).

Schuhardt et al. showed that degradation products of the amino acid, cystine, caused toxicity in certain batches of dehydrated peptones. The elemental sulfur produced from autoclaved cystine solutions were then neutralized by the addition of blood or serum (96). Some fatty acids were also shown to be toxic to brucellae (7, 41). Anti-brucella factors were found in some batches of peptone after filtration through cotton-wool, which had been released from cotton. Huddleson reported that toxic factors were neutralized after treatment with charcoal,

serum and Tween 40 (41). Additives such as serum, dextrose, or glycerol to peptone media were shown to enhance the growth of certain strains of Brucella by correcting for inconsistent muscle sugar content in peptone digest (42).

Chemically defined synthetic media were successful only after the nutritional requirements of Brucella were determined, especially the need for accessory factors. Different amino acids were used for nitrogen sources in media, however the addition of supplements revealed the most significant affect on the growth of B. abortus. Supplements incorporated into media included: biotin, calcium panthenate, nicotinic acid, thiamine chloride, and hemin (35, 58, 67, 90).

Basal media currently used are described in Alton et al. which include; serum-dextrose agar, tween-dextrose agar, glycerol-dextrose agar, potato infusion agar, and dehydrated forms of trypticase soy and tryptose agar (1). A comparison of the efficiency of these basal media was made revealing that only serum-dextrose agar was capable of supporting the growth of all strains examined including B. abortus biotype 2 (76). Sang et al., reported Schaedler Agar[1] containing cystine and hemin superior to Brucella Agar[1] as a basal medium for brucella isolations (94).

Development of Selective Media

The development of a selective medium for brucellae was highly significant because it permitted isolation by direct culture from

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[1]Baltimore Biologic Laboratory, Cockeysville, MD.

contaminated material such as milk, soil, feces, and tissues. Various dyes, chemicals, and antibiotics have been incorporated into the currently recommended selective media.

Liver infusion agar containing gentian violet 1:100,000 was the first selective medium employed for the isolation of B. abortus (40). This medium was reported to be as efficient as guinea pig inoculation procedures. Gould and Huddleson, as sited by Robertson, suggested that a decrease of the concentration of gentian violet to 1:200,000 resulted in a less inhibitory effect to some brucellae (89). Other dyes, malachite green and victoria blue were investigated, but no advantage over gentian violet was found (89).

A significant improvement in the selectivity of brucella culture media was obtained with the introduction of antibiotic preparations. A medium for B. suis incorporating the antibiotic tryrothricin and sodium azide in tryptose agar base was described (89). A selective medium which enabled the isolation of brucella in almost pure culture from feces, contained bacitracin, sulfadiazine, polymyxin D, and circulim in tryptose agar (89). The addition of other inhibitory agents in selective media included; cyclohexiмide, useful in the control of yeast and fungus contamination, penicillin, and crystal violet (55, 63). Crystal violet was later replaced by ethyl violet which was more selective and possessed more reliable antibacterial properties.

The potent antibacterial activity of nitrofuran compounds was examined in Morris medium. However, Morris medium required longer incubation periods up to 10 to 12 days, with a reduced colony size. The addition of erythritol to Morris medium enhanced the growth rate of certain strains

of B. abortus while it was inhibitory to others (47, 48, 108).

Serum-dextrose agar containing the antibiotics polymyxin, bacitracin, and cycloheximide, supported the growth of all strains of B. abortus, B. melitensis, and B. suis (76, 113). Brucella biotype 2 proved to be sensitive to selective media containing dyes and amphotercin B (29, 78).

Ryan developed a highly selective medium which contained penicillin, polymyxin B, ristocetin, nalidixic acid, cetrimide, cycloheximide, and nystatin for isolation of B. abortus from milk (93).

A selective medium for the isolation of B. ovis from ram semen was described as a modification of Thayer-Martin medium by omission of IsoVitale X[1] and the addition of antimicrobial substances; vancomycin, colistemethate, nystatin, and furadantin used to control the natural flora of ram semen (10).

Farrell determined the sensitivity of 105 strains of Brucella to a number of antimicrobial agents (29). His results revealed amphotericin B inhibited all strains of biotype 2 and the dye-sensitive strains of biotype 4; so consequently it was considered unsuitable as a selective agent for the isolation of B. abortus. It was considered that colistin was no more selective than polymyxin B and its use as a selective agent offered no advantages over polymyxin B. Farrell developed a medium which consisted of serum-dextrose agar with bacitracin, vancomycin, polymyxin B, nalidixic acid, nystatin, and cycloheximide and concluded that these

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[1]Baltimore Biologic Laboratory, Cockeysville, MD.

inhibitors effectively suppressed the growth of contaminating organisms without inhibiting the growth of Brucella, including biotype 2.

Farrell and Robertson compared various media for the isolation of Brucella from milk. For certain selective media, isolation rates were equal to results of the guinea pig inoculation method (30). Although Farrell's medium and Ryan's medium had similar isolation rates, Ryan's medium inhibited B. abortus biotype 2. Serum-dextrose agar and Mair's medium containing bacitracin, polymyxin and cycloheximide were not able to suppress all contaminating organisms and lower isolation rates for B. abortus were observed.

A comparative study revealed Farrell's medium was more efficient than either serum-dextrose agar or Barrow and Peel's medium with erythritol for the isolation of B. abortus from milk and vaginal mucus (43).

Brodie and Sinton used a fluid enrichment medium for growth of B. abortus from milk (9). Its use increased the recovery rate 10-16% over that reported for serum-dextrose agar or Ryan's medium (9). However, a recent study by Berkhoff and Nicoletti revealed that a modification of Brodie and Sinton's fluid medium was inferior to Brucella Agar containing bacitracin for isolation of B. abortus from milk (5).

Culture Media Additives

The value of sodium polyanethol sulfonate (SPS) as an additive in blood culture media is widely recognized. Several studies of blood culture systems have demonstrated improved survival, increased frequency of

isolation, and more rapid recovery of most bacteria in media with SPS compared to media lacking the additive (4, 23, 26, 91, 92, 112). SPS was shown to be inhibitory to certain anaerobic bacteria including Peptostreptococcus anaerobius and Neisseria meningitidis (25, 27, 36, 39, 51), but recent studies have shown this effect to be medium-dependent (23, 24, 88, 115).

Sodium amylosulfate (SAS) was developed as an additive for blood culture media to avoid the problem of an inhibitory effect on growth of anaerobic bacteria (54). SAS has been shown to be as effective as SPS as an inhibitor of both the cellular and humoral antimicrobial systems of blood (2, 4, 26, 32, 37, 51, 53, 54, 91, 111, 112).

SAS and SPS have many similar properties which account for the same modes of action as an additive in culture media. They are synthetic polyanionic anticoagulants with the following properties: anticomplementary, antiphagocytic, and are unchanged by heating or by the action of dilute acids or alkalis. They act in a manner similar to other aromatic compounds of high molecular weight by the interaction of highly negative charged sites of the polyanion with the positively charged sites of other molecules (28, 32, 52, 53, 112). The polyanions have been shown to interact with at least three major antibacterial components existing in human serum that affects the growth of organisms in blood: 1) B lysin, 2) normal antibody-complement system, 3) lysozyme or muramidase (4, 14, 28, 31, 61, 104, 106, 107). It has been demonstrated that SPS prevents the killing of serum-sensitive strains of Escherichia coli (E. coli) by

normal serum (4, 14, 21). SPS and SAS have been shown to form insoluble precipitates with lysozyme, thereby inhibiting its enzymatic action. However, a recent study reports complete inactivation of lysozyme by SPS, but not SAS (4, 53). SPS was found to precipitate other human plasma components such as betaglobulins, fibrinogen, pooled gamma globulins, and beta lipoproteins (4, 52).

Polyanions have been shown to inhibit the classical and alternate complement pathways through activation and binding of C1 and C3 components. SPS was demonstrated to interfere with the components of the classical pathway in at least three different ways; 1) SPS binds directly to C1q, a subunit of the first component of complement, and the binding site of $C\bar{1}s$ to antibody-antigen complex, 2) inhibits the binding of C2 to C4 by sequestering the Mg^{+2} ions, 3) SPS prevents the consumption of C4 and C2 by $C\bar{1}s$, by interfering with its' C4 and C2 binding sites (53, 59, 60, 106, 107).

It has been observed that SPS and SAS do not completely inhibit serum agglutinins. Therefore, it is assumed that the binding of complement by polyanions is of major importance in the reduction of antimicrobial activity of serum. Furthermore, SPS has been shown to inhibit complement-dependent killing of certain organisms. SPS does not inhibit the metabolic activity of leukocytes, therefore if phagocytosis occurs, SPS would not be expected to prevent the killing of microorganisms which are intracellular (4, 111).

No information was available on the use of SPS or SAS to inhibit the antibacterial action in other body fluids or excretions in order to increase the recovery rate of bacteria for isolation.

PART 1. PRELIMINARY STUDIES OF THE DEVELOPMENT OF A SELECTIVE ENRICHMENT BROTH

MATERIALS AND METHODS

Organisms

Brucella abortus strain 19 vaccine culture and *B. abortus* strain 2308, the USDA challenge strain, were obtained from the USDA.[1]

The following organisms were obtained from the Department of Microbiology and Preventive Medicine, Iowa State University:

- *Staphylococcus aureus* ATCC2593
- *Escherichia coli* ATCC25922
- *Bordetella bronchiseptica*
- *Pasteurella multocida*
- *Pseudomonas aeruginosa* strain A
- *Pseudomonas aeruginosa* strain O
- *Corynebacterium pseudotuberculosis*
- *Corynebacterium equi*
- *Bacillus cereus*
- *Klebsiella pneumoniae*
- *Proteus mirabilis*
- *Salmonella gallinarum*
- *Salmonella minnesota*
- *Streptococcus uberis*
- Yeast (unidentified)
- *Canidida albicans*
- *Enterobacter aerogenes*

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[1] National Veterinary Services Laboratories (NVSL), USDA, Ames, IA.

Culture Media

Solid medium

Tryptose agar plates (TA)[1] with 5% bovine serum[2] were prepared as previously described (1).

Liquid media

The following broth media: trypticase soy broth (TSB),[1] tryptose broth (TB),[1] brucella broth (BB),[3] and 1% peptone broth diluent;[4] were prepared as described by the manufacturer, and autoclaved at 121° C for 15 minutes with the pH adjusted to 7.0.

A description of the special medium preparations and the sources used for the following broths are shown in Table A2: special tryptose broth (STB), GC broth, ANM broth, enrichment broth No. 1 (EB1), and enrichment broth No. 2 (EB2).

The coding of these media and the added components are shown in Table 1.

Culture Media Additives

Hemoglobin[1]

A one percent stock solution of hemoglobin was made by gradually adding 200 ml of distilled water to two grams of dry hemoglobin.

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[1] Difco Laboratories, Detroit, MI.

[2] National Veterinary Services Laboratories (NVSL), USDA, Ames, IA.

[3] Pfizer Diagnostics Division, N. Y., NY.

[4] Protease No. 3, Difco Laboratories, Detroit, MI.

The solution was autoclaved at 121° C for 15 minutes and stored at 4° C.

Hemin[1]

A hemin stock solution was prepared by dissolving 50 mg of recrystallized hemin in 1 ml of 1N NaOH. To this, 99 ml of distilled water was added. The solution was sterilized by autoclaving at 121° C for 15 minutes and stored at 4° C.

Vitamin K[2]

A stock solution was prepared as recommended by the manufacturer, filter sterilized and stored at 4° C.

Bovine serum[3]

Stored at 4° C.

Sodium amylosulfate (SAS)[4] and sodium polyanethol sulfonate (SPS)[1]

A ten percent stock solution was prepared by dissolving 1 gram of SAS or SPS in 10 ml distilled water. The solution was autoclaved at 121° C for 15 minutes and stored at 4° C.

Inhibitory agents

Stock solutions of each inhibitory agent shown on Table 2 were prepared as recommended by the manufacturer and stored at 4° C. The concentrations tested were prepared by a series of twofold dilutions of the stock solution in sterile tryptose broth.

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[1]Eastman Kodak Chemical Co., Rochester, NY.

[2]ICN Pharmaceuticals, Cleveland, OH.

[3]National Veterinary Services Laboratories (NVSL), USDA, Ames, IA.

[4]Searle Laboratories, Chicago, IL., Lot #nH4.

Table 1. Composition and coding of media and added components

Media code	Basal medium	Ingredients[a]		
		Hemoglobin	Hemin	Vitamin K
TB	Tryptose broth	-[d]	-[e]	-
TB-H	"	-	+	-
TB-1	"	-	-	-
TB-1-H	"	-	+	-
TB-2	"	-		-
TB-2-H	"	-	+	-
TB-2-H-SAS	"	-	+	-
STB[f]	Special tryptose broth	-	-	-
STB-G	"	-	-	-
STB-G-H-V	"	-	+	+
ANM[f]	ANM broth	-	-	-
ANM-S	"	-	-	-
ANM-H	"	-	+	-
ANM-H-V	"	-	+	+
BB	Brucella broth	-	-	-
BB-S	"	-	-	-
BB-H-V	"	-	+	+
BB-S-H-V	"	-	+	+
GC	GC broth	+	-	-

[a]Concentration used: hemoglobin (2%), hemin (5 ug/ml), vitamin K (.01 mg/ml), glucose (5%), SAS (.05%).

[b]Inhibitors-1 includes: cycloheximide (100 ug/ml), nalidixic acid (5 ug/ml), and vancomycin (25 ug/ml).

[c]Inhibitors-2 includes: bacitracin (25 ug/ml), cycloheximide (100 ug/ml), nalidixic acid (5 ug/ml), polymyxin B sulfate (5 units/ml), and vancomycin (25 ug/ml).

[d]- = ingredient not included.

[e]+ = ingredient included.

[f]Prepared as shown in Table A2.

Ingredients				
Bovine serum (5%)	Glucose	Inhibitors-1[b]	Inhibitors-2[c]	SAS
-	-	-	-	-
-	-	-	-	-
-	-	+	-	-
-	-	+	-	-
-	-	-	+	-
-	-	-	+	-
-	-	-	+	+
-	-	-	-	-
-	+	-	-	-
-	+	-	-	-
-	+	-	-	-
+	+	-	-	-
-	+	-	-	-
-	+	-	-	-
-	-	-	-	-
+	-	-	-	-
-	-	-	-	-
+	-	-	-	-
-	-	-	-	-

Inocula Preparation

Five colonies of each organism to be tested were inoculated into 5.0 ml tryptose broth, and incubated for 18 hours at 37° C. All cultures, except B. abortus, were diluted in phosphate buffered saline (PBS) and contained approximately 10^5 to 10^6 viable cells per ml. The 18 hour cultures of B. abortus strains 19 and 2308 were diluted 1:100 by adding a 0.1 ml of culture into 9.9 ml of sterile PBS.

Antibiotic Susceptibility Testing Method

Five tenths ml of each concentration of the antibiotics tested was delivered into four 13 X 100 mm test tubes (Table 2). Four additional tubes received 0.5 ml of tryptose broth not containing antibiotic served as controls. Suspensions of the following organisms; B. abortus strain 19, strain 2308, Staphylococcus aureus (Staph. aureus), and E. coli were prepared as described above. Five hundredths of each suspension was inoculated into one tube containing the antibiotic and one control tube.

All tubes were incubated at 37° C and then observed for the presence of growth at 24 and 48 hours.

Tolerance of B. abortus to SAS and SPS

The procedure used for determining the effects of various concentrations of SAS and SPS on the growth of B. abortus is described in Table 3. A drop (0.05 ml) of inoculum of strains 19 and 2308, prepared as described above, was added as indicated. The tubes were then incubated for 72 hours at 37° C after which each tube was observed for the presence of growth.

Table 2. List of inhibitory agents, stock solutions prepared, and concentrations tested

Inhibitory agent	Stock solution concentration ug/ml	Concentrations tested ug/ml	Source
Amphotericin B	80[a]	40, 20, 10, 5, 2.5	Squibb[b]
Bacitracin	160[a]	80, 40, 20, 10, 5[a]	Squibb[b]
Cycloheximide	500[a]	250, 125, 62.5, 31.25	Upjohn[d]
Cycloserine	2400[a]	1200, 600, 300, 150, 75	ICN[e]
Nalidixic acid	80[a]	40, 20, 10, 5, 2.5	Aldrich[f]
Nystatin	280[c]	140, 70, 35, 17.53, 8.8[a]	ICN[e]
Polymyxin B sulfate	76.8[c]	38, 19.2, 9.6, 4.8, 2.4[a]	Pfizer[g]
Vancomycin	200[a]	100, 50, 25, 12.5	EliLily[h]

[a]Concentration in ug/ml.

[b]Squibb Pharmaceuticals, Princeton, NY.

[c]Concentration in units/ml.

[d]Upjohn Co., Kalamazoo, MI.

[e]ICN Pharmaceutical Co., Cleveland, OH.

[f]Aldrich Chemical Co., Milwaukee, WI.

[g]Pfizer Diagnostics Division, N. Y., NY.

[h]Eli Lilly, Indianapolis, IN.

Table 3. Protocol to test the tolerance of B. abortus strains 19 and 2308 to various concentrations of SAS and SPS

Concentration SAS or SPS in 2 ml tryptose broth	Inoculum (ml)	
	Strain 19	Strain 2308
0.01%	0.05	0.05
0.05%	0.05	0.05
0.1%	0.05	0.05
0.5%	0.05	0.05
none	0.05	0.05
none	0	0

Surface Viable Count Method

Viable counts were determined by the method of Miles and Misra with minor modifications (75) on serum tryptose agar plates which had been dried at 37° C for 24 hours before use. Such plates would absorb 0.04 ml of inoculum in 15-20 minutes. Tenfold serial dilutions of the culture to be tested were made in 1% peptone diluent. On dried plates, 0.04 ml of the diluted culture was dropped from a sterile 1.0 ml serological pipette (delivering 25 drops/ml) held at approximately 2 cm above the surface.

Duplicate dilutions of each culture were made and triplicate plates received one drop of dilution in a numbered section. After absorption of the drops, the plates were incubated at 37° C for 3-4 days. Counts were made in drop areas with the largest number of colony-forming units which could be accurately counted without signs of colony overcrowding.

The colony count was estimated from the mean of six counts. The average number of colonies in the drop multiplied by 25 times the dilution factor for the drop gives the number of viable bacteria per milliliter in the culture examined.

Preparation and Maintenance of the Standard *B. abortus* Strain 19 Control Culture

Brucella abortus strain 19 lyophilized vaccine[1] was reconstituted with 5 ml of saline. One ml of strain 19 suspension was used to inoculate 5 mls of tryptose broth (TB), incubated at 37° C for 24 hours. A loopful of the culture was inoculated onto a tryptose agar plate (TA), and incubated at 37° C for 48 hours. Ten colonies were selected, and transferred to 9.9 ml of trypticase soy broth (TSB), incubated for 18 hours at 37° C. The TSB culture was vortexed for 5 seconds, and 0.1 ml was then transferred to 9.9 ml TSB. Daily 18 hour subcultures were made by the procedure described above. A viable plate count was made each day to determine if the number of viable organisms remained constant throughout the testing period.

Evaluation of Various Liquid Media for Optimal Growth of *B. abortus*

Duplicate samples of each broth were tested as listed in Table 1. To each sample containing 9.9 ml of liquid medium, 0.1 ml of the standardized strain 19 inoculum was added. Broths were incubated at 37° C for 24 hours. Viable plate counts were made at 0, 12, and 24 hours.

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[1]National Veterinary Services Laboratories (NVSL), USDA, Ames, IA.

RESULTS

Susceptibility Testing to Inhibitory Agents

The sensitivity profiles for B. abortus strains 19 and 2308, Staphylococcus aureus, and Escherichia coli to selected inhibitory agents are shown in Figure 1. The results revealed a characteristic growth pattern for the organisms subjected to the inhibitory agents tested. Polymyxin B sulfate appeared slightly inhibitory toward strains 19 and 2308 at a minimal inhibitory concentration of 9.6 units per ml. Antifungal agents, cycloheximide and nystatin, did not prevent the growth of strains 19 and 2308 at the concentrations tested (Table A3).

Tolerance of B. abortus for SAS and SPS

No inhibitory effect was observed on the growth of strains 19 and 2308 by the concentrations of SAS or SPS tested as shown in Table 4.

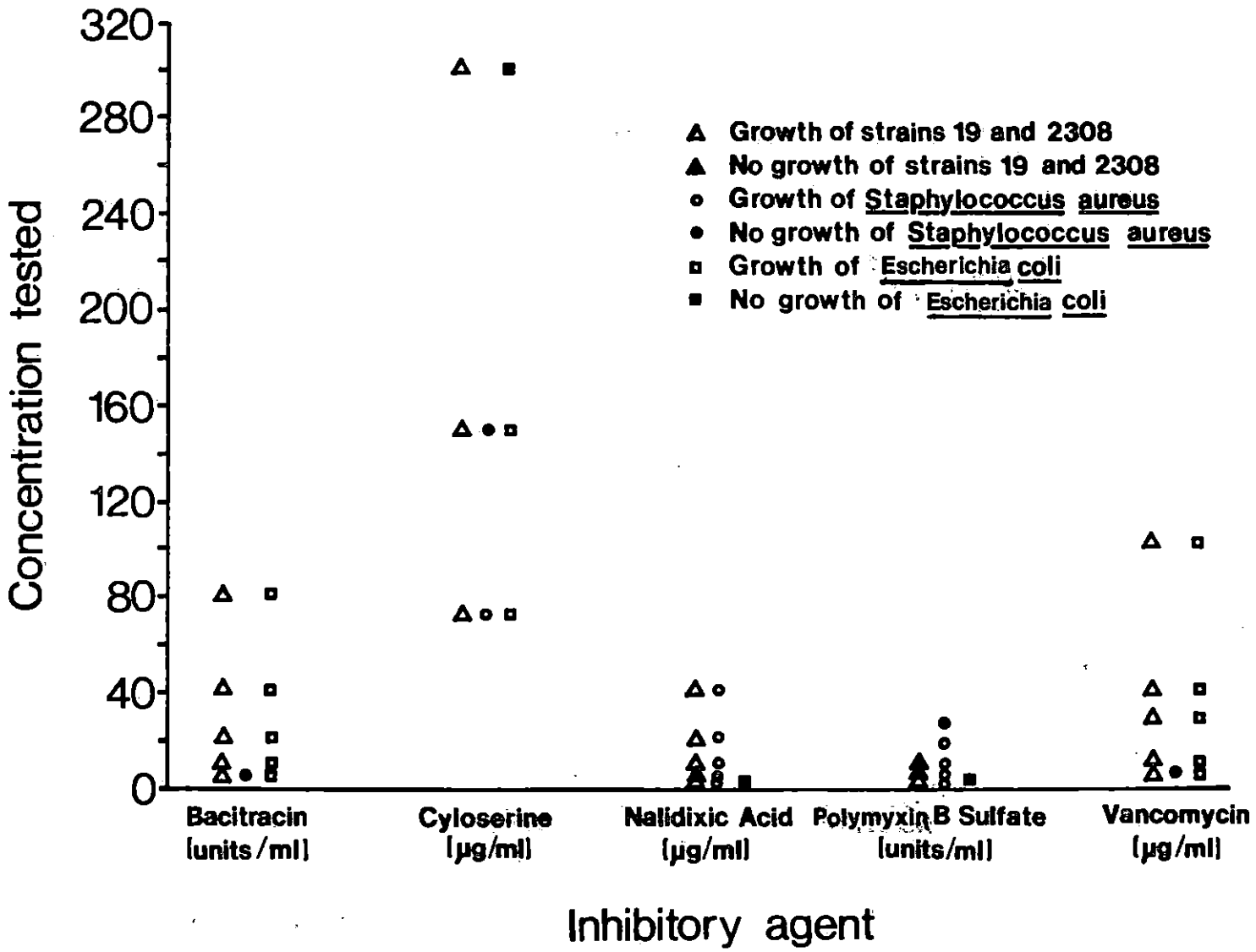
Table 4. Tolerance of strains 19 and 2308 to various concentrations of SAS and SPS

Chemical	Organisms	Concentration (%)					Recommended[a] concentrations %
		0.5	0.1	0.05	0.01	Control	
SAS	Strain 19	+[b]	+	+	+	+	.05
	Strain 2308	+	+	+	+	+	
SPS	Strain 19	+	+	+	+	+	.05
	Strain 2308	+	+	+	+	+	

[a]Recommended concentrations described previously by (37, 52, 54).

[b]+ = presence of growth.

Figure 1. Sensitivity profiles of Brucella abortus strains 19 and 2308, Staphylococcus aureus, and Escherichia coli using various inhibitory agents


320
280
240
200
160
120
80
40
0
Concentration tested
Growth of strains 19 and 2308
No growth of strains 19 and 2308
Growth of Staphylococcus aureus
No growth of Staphylococcus aureus
Growth of Escherichia coli
No growth of Escherichia coli
Bacitracin (units/ml)
Cycloserine (µg/ml)
Nalidixic Acid (µg/ml)
Polymyxin B Sulfate (units/ml)
Vancomycin (µg/ml)
Inhibitory agent

Evaluation of Media

A number of media formulations which have been recommended for the growth of brucella were evaluated using strain 19 in preliminary trials 1 and 2. The medium which provided for the optimum growth of strain 19 was then selected as the medium base into which various inhibitory agents were incorporated in order to determine their usefulness in a selective medium.

The results of preliminary trial 1 indicated STB was superior to TB, ANM, and BB as a basic medium without additives for the growth of strain 19 (Table 5). Further evaluation of TB, STB, and ANM media with the addition of hemin, vitamin K, and glucose were made in preliminary trial 2 (Table 6). Upon repeated studies TB appeared to be superior to STB for the growth of strain 19.

The addition of hemin to each medium revealed an increased growth index above that of the basal medium without additives. The addition of hemin and vitamin K to ANM had a stimulatory effect approximately equal to the growth response observed in GC broth containing hemoglobin (Table 5). TB-H demonstrated the highest growth index of media tested in this preliminary study. When glucose was added to STB a stimulatory effect was not observed. The combination of glucose, hemin, and vitamin K added to STB was observed to have only a minor stimulatory effect. The addition of 5% bovine serum did not reveal a significant effect on the growth response of strain 19. TB-H was chosen as the basal medium for further study.

The growth indices of strain 19 grown in tryptose broth containing

Table 5[a]. Preliminary Trial 1 - The growth indices of strain 19 grown in various basic media, and media with selected additives

Liquid medium	Growth index[b] (24 hours)
TB = tryptose broth	129.4
STB = special tryptose broth	157.8
ANM = ANM broth	111.7
ANM-S = ANM broth plus bovine serum	122.9
ANM-H-V = ANM broth plus hemin and vitamin K	148.0
BB = brucella broth	87.5
BB-S = brucella broth plus bovine serum	30.8
BB-H-V = brucella broth plus hemin and vitamin K	70.8
BB-S-H-V = brucella broth plus bovine serum, hemin and vitamin K	21.7
GC = GC broth with hemoglobin	152.6

[a]Complete results listed in Table A4.

[b]Growth index = $\frac{\text{viable colony count at 24 hours}}{\text{viable colony count at 0 hour}}$.

Table 6[a]. Preliminary Trial 2 - The growth indices of strain 19 grown in various basic media, and media with selected additives

Liquid medium	Growth index[b] (24 hours)
TB = tryptose broth	121.4
TB-H = tryptose broth plus hemin	135.0
STB = special tryptose broth	103.7
STB-G = special tryptose broth plus glucose	88.9
STB-G-H-V = special tryptose broth plus glucose, hemin, and vitamin K	114.3
ANM-H = ANM broth plus hemin	110.0

[a]Complete results listed in Table A5.

[b]Growth index = $\frac{\text{viable colony count at 24 hours}}{\text{viable colony count at 0 hour}}$.

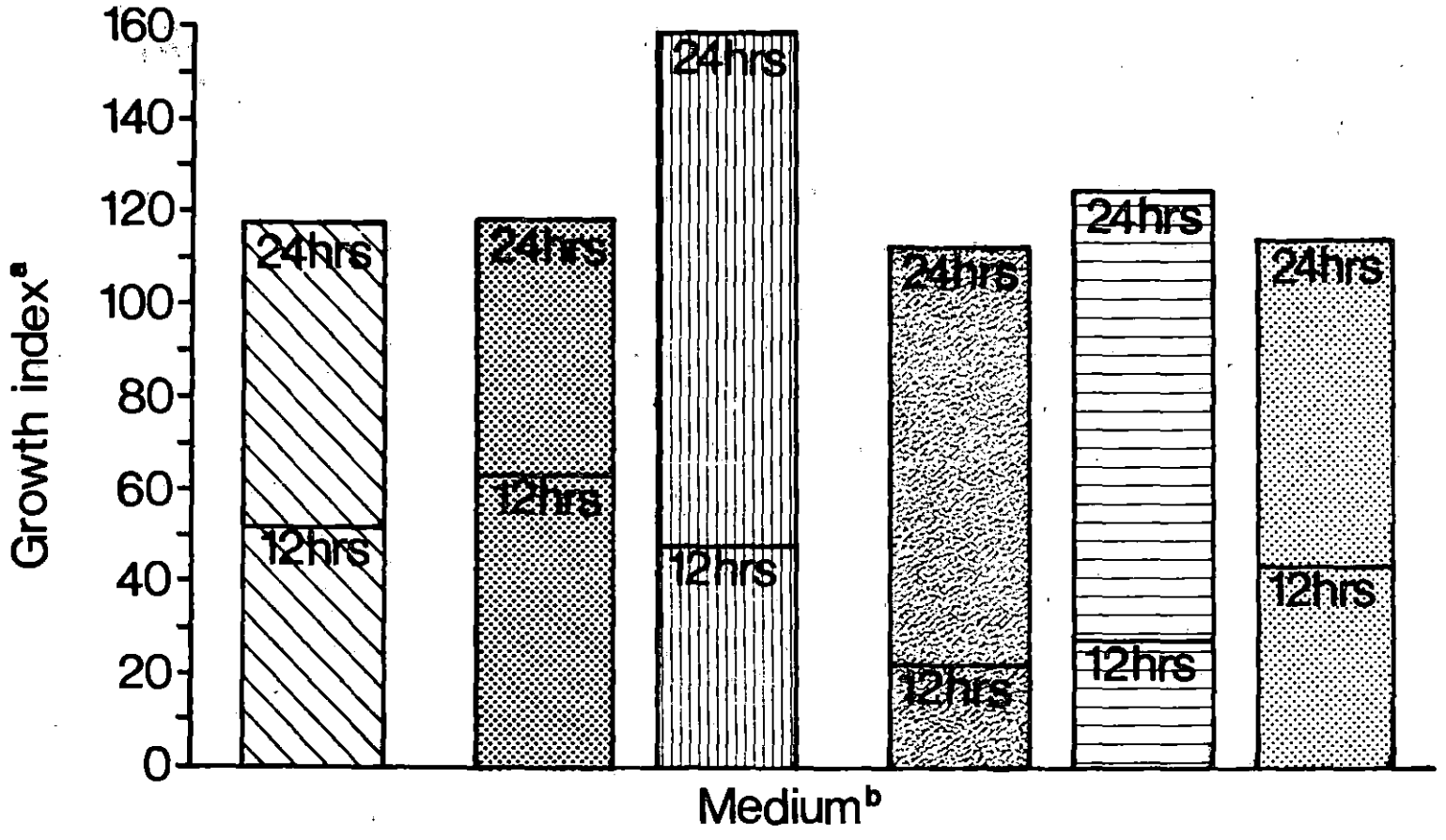
inhibitors-1 (vancomycin, nalidixic acid, and cycloheximide) and inhibitors-2 (vancomycin, nalidixic acid, cycloheximide, polymyxin B sulfate, and bacitracin) are shown in Figure 2. When comparing the growth index for inhibitors-1 and -2 in TB at 12 hours, a marked difference (40,6) was observed, whereas at 24 hours there was no difference. With the addition of hemin to both inhibitors-1 and -2 in TB a minor difference was observed at 12 hours and a major difference (33.9) appeared at 24 hours. The addition of hemin to TB-1 produced the highest growth index of 158.9 observed at 24 hours.

The addition of SAS to inhibitors-2 with hemin showed a slight advantage at 12 hours, and revealed a slight decrease at 24 hours when compared to the same medium without SAS.

Control of Non-brucella Organisms by Inhibitors

The ability of inhibitors-1 and -2 in tryptose broth to inhibit the growth of various non-brucella organisms while permitting the growth of strains 19 and 2308 is shown in Table 7. TB containing only inhibitors-2 was capable of inhibiting all the non-brucella organisms except Proteus mirabilis. The addition of hemin to TB containing inhibitors-2 also permitted the growth of Pseudomonas aeruginosa in addition to Proteus mirabilis. The medium containing inhibitors-1 with and without hemin showed less inhibition allowing the growth of six additional organisms. SAS did not appear to affect the action shown by inhibitors-2 when combined with hemin.

Figure 2. Growth indices of B. abortus strain 19 grown in tryptose broth containing different inhibitory agents, hemin, and SAS



[a] Growth index = $\frac{\text{viable colony count at 12 or 24 hours}}{\text{viable colony count at 0 hour}}$

[b] Medium

- Tryptose broth
- Tryptose broth plus inhibitors #1
- Tryptose broth plus inhibitors #1, and hemin
- Tryptose broth plus inhibitors #2
- Tryptose broth plus inhibitors #2, and hemin
- Tryptose broth plus inhibitors #2, hemin, and sodium amylosulfate

Table 7. The ability of inhibitory agents with and without hemin and SAS additives to inhibit the growth of respective organisms and B. abortus strains 19 and 2308

Organism	Medium				
	TB-1	TB-2	TB-1-H	TB-2-H	TB-2-H-SAS
Strain 19	+[a]	+	+	+	+
Strain 2308	+	+	+	+	+
Yeast (unidentified)	-[b]	-	-	-	-
Canidida albicans	-	-	-	-	-
Bordetella bronchiseptica	+	-	+	-	-
Pseudomonas aeruginosa (A)[c]	+	-	+	+	+
Pseudomonas aeruginosa (O)[d]	+	-	+	+	+
Corynebacterium pseudotuberculosis	-	-	-	-	-
Corynebacterium equi	-	-	-	-	-
Bacillus cereus	-	-	-	-	-
Klebsiella pneumoniae	+	-	+	-	-
Proteus mirabilis	+	+	+	+	+
Salmonella gallinarum	+	-	+	-	-
Salmonella minnesota	-	-	-	-	-
Enterobacter aerogenes	+	-	+	-	-
Streptococcus uberis	-	-	-	-	-
Staphylococcus aureus	+	-	+	-	-
Escherichia coli	+	-	+	-	-
Control	-	-	-	-	-

[a] + = presence of growth.

[b] - = no growth.

[c] Pseudomonas aeruginosa strain A.

[d] Pseudomonas aeruginosa strain O.

DISCUSSION

A selective enrichment medium was developed for the isolation of B. abortus from bovine vaginal secretions. The results suggested that tryptose broth with the addition of hemin was the most satisfactory for the growth of strain 19 in the various broths compounded (Table 6). Libberman also reported that hemin was superior to a number of supplements for increasing the colony counts of B. abortus, B. suis, and B. melitensis (58). The significance of hemin was also observed by Sang et al. using Schaedler medium containing cystine and hemin; a 20% greater isolation rate of B. abortus from milk was observed on this media than on a routinely used brucella agar (94).

Increased recovery rates of B. ovis from the semen of rams compared to the recovery rates made on serum tryptose agar with antibiotics have been obtained on modified Thayer-Martin medium containing hemoglobin (10). No important differences were observed in the number of strain 19 isolations between media to which hemin and hemoglobin were added (Table 5). A vitamin K-hemin supplement has been recommended for growth of fastidious anaerobes; however, it did not appear to increase the number of strain 19 colonies as compared to media with hemin alone. Hemin was selected as the enrichment supplement rather than hemoglobin or vitamin K-hemin because there was no important differences in number of colonies of strain 19. Moreover, it is simple to prepare and store, and is more soluble in water resulting in a clear preparation in which contamination is more readily detected. The addition of 5% serum to media did not appear to

increase the growth of strain 19. Therefore, it was not added to media to be further evaluated in this study, since strains 19 and 2308 do not require serum for growth. It should be emphasized that when enrichment broth is used for the isolation of field strains of B. abortus, 5% bovine serum must be added since it is required for the growth of B. abortus biotype 2. A report indicated that enrichment of media with serum was necessary for maximum isolation of all strains of Brucella (78). An important difference was also observed between media with and without serum when infected tissues were the source of Brucella rather than when laboratory propagated strains were used.

Discrepancies between the results of the growth indices of strain 19 in preliminary trials 1 and 2 were observed (Tables 5 and 6). However, the number of tests conducted were not determined statistically significant. Trial 1 yielded different growth indices for the broths tested than in study 2, in which tryptose broth was observed as the control medium. Possible differences may have been caused by interaction of different media components with the various enrichment added resulting in synergistic and antagonistic effects.

Of the combination of inhibitory agents added to enrichment broth, inhibitors-2 (bacitracin, cycloheximide, nalidixic acid, polymyxin B sulfate, and vancomycin) was found to be more efficient in controlling non-brucella organisms than inhibitors-1 (cycloheximide, nalidixic acid, and vancomycin) (Table 7). These results are consistent with those reported by Hunter and Kearns who used Farrell's medium containing inhibitors-2 plus nystatin for effective suppression of contaminants in

milk and vaginal mucus samples (30). Thus, Farrell's medium provided for an improved recovery rate of B. abortus as compared to that of serum dextrose agar (bacitracin, polymyxin B, and naramycin) and Barrow and Peel medium (bacitracin, polymyxin B, vancomycin, and cyclohexamide) (43).

The addition of hemin to TB-1 appeared to increase the number of colonies of strain 19 to a greater extent than in TB-2 (Figure 2). It was observed that TB-2 was effective in inhibiting the growth of Pseudomonas while TB-2-H was not (Table 7). It was found that the addition of hemin to TB-2 appeared to be less effective in inhibiting Pseudomonas and in promoting the growth of strain 19. This suggests an incompatibility between hemin and inhibitors-2, providing a loss of both beneficial results. A phenomenon similar to this was reported when testing selective hemoglobin-containing media for the isolation of Neisseria gonorrhoeae (100). It was suggested that hemoglobin was binding some of the vancomycin, allowing for the growth of gram-positive contaminants. In the presence of hemoglobin, a higher concentration of vancomycin was required to inhibit four strains of Staphylococcus epidermidis. In this study using strain 19, the activity of polymyxin B sulfate may have possibly been reduced by the binding of hemin in TB-2-H.

Cycloheximide appeared to efficiently suppress fungi tested (Table 7); therefore, it was included as one of the inhibitors in the final formulation of the selective broth.

No toxic effects to strains 19 or 2308 were observed with the addition of SAS to TB. Also, no important effects were demonstrated in the ability of inhibitors-2 to control non-brucella organisms with the

addition of SAS in TB-2-H-SAS. Therefore, the addition to the selective enrichment broth of SAS could be made because it did not inhibit the growth of B. abortus. An extension of this study in Part 2 will be to obtain information on the ability of SAS to interfere with the antimicrobial activity of the vaginal secretions.

PART 2. THE COMPARISON OF THE ENRICHMENT BROTH AND DIRECT STREAK PLATE METHODS USED TO DETERMINE SHEDDING PATTERNS OF *B. ABORTUS* IN THE VAGINAL SECRETIONS OF ADULT CATTLE

MATERIALS AND METHODS

B. abortus Strains

Brucella abortus strains 19 and 2308 control cultures were obtained from the USDA.[1]

B. abortus Strain 19 Vaccine

Brucella abortus strain 19 vaccine lot was prepared by the USDA[1] from the original strain 19 seed culture as previously described (110). Special preparations of the strain 19 vaccine were made yielding the required volumes and containing the number of viable organisms/ml shown in Table 8.

B. abortus Strain 2308 Challenge Dose Preparation

The challenge inoculum containing 8.2×10^5 viable organisms was prepared by USDA[1] from a lyophilized culture of strain 2308.

Experimental Animals

Ninety-six pregnant beef cows with no previous known exposure to B. abortus were randomly assigned to six groups and vaccinated subcutaneously with strain 19 vaccine as shown in Table 8. Sixteen weeks after vaccination, each animal was challenged by inoculation of 8.2×10^5 strain 2308 by the conjunctival route. Each animal was housed in a separate stall.

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[1]National Veterinary Services Laboratories, USDA, Ames, IA.

Table 8. Vaccination schedule for comparison of different dosage levels of B. abortus strain 19

Group	No. animals	Eartag Nos.	Vaccine dosage	
			Volume	No. viable organisms
V1	15	25-39	5 ml	7.8×10^{10}
V2	15	40-50, 106 52-54	2 ml	5.0×10^{9}
V3	16	55-62, 119 63-67, 116, 69	2 ml	9.2×10^{8}
V4	15	16, 17, 124 19-24, 70-72 93-95	2 ml	1.4×10^{8}
V5	15	1-15	2 ml	2.3×10^{7}
C6	20	73-92	none	none

Sampling Schedule

A vaginal sample was collected and cultured for B. abortus on the day of challenge, and at weekly intervals thereafter, until three consecutive negative results were obtained after abortion or parturition. Additional samples were collected from animals; 1) prior to parturition, 2) within 48 hours after abortion or parturition, and 3) when repeated isolations of B. abortus were made from the vaginal discharges. Duplicate samples were collected at various intervals.

Collection of Vaginal Samples

The vulvular region of the cow was wiped clean by an isopropyl alcohol[1] saturated pledget of cotton. A sterile, disposable culture swab enclosed in a plastic sheath[2] was inserted into the vagina and the swab was then extended about ten inches into the vagina. The vaginal secretions were absorbed for 10-15 seconds. The swab was withdrawn about halfway into the external sheath and the entire swabbing instrument removed. The external plastic sheath was cut with a disinfected snipper approximately two inches distal to the inner swab. The swab was pushed through the protective sheath and inserted into the sterile culture tube containing 0.5 ml of tryptose broth. The vaginal swab was cut off and the tube recapped, and then transferred to the laboratory. After each animal was sampled, hands and boots of personnel were disinfected in Microbac disinfectant[3] before entering the next cow stall. Samples were processed approximately three-hours after collection.

Culture Media

Solid media used for the isolation of B. abortus from the vaginal secretions by the direct streak method included; tryptose serum agar with antibiotics (TSA), and tryptose serum agar with antibiotics and ethyl violet (EVTSA).

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[1]Mallinckrodt, Inc., Paris, KY.

[2]Kalayjian Industries, Long Beach, CA.

[3]Econ Laboratories, St. Paul, MN.

TSA contained: Difco tryptose agar,[1] 5% bovine serum,[2] 30 ug/ml cycloheximide,[3] 7.5 ug/ml bacitracin,[4] and 1.8 units/ml polymyxin B sulfate.[5]

EVTSA contained the same materials as listed for TSA with the addition of 140 ug/ml ethyl violet dye.[6] These media were prepared as previously described (1).

Enrichment broth medium No. 1 and No. 2 were prepared as described in Part 1.

Cultural Methods

Each vaginal swab was directly streaked over the surface of one TSA plate (medium 1) and one EVTSA plate (medium 2). The cotton tip of the swab was then cut off with sterile scissors, placed in the enrichment broth, and incubated at 37° C for 5 to 7 days. Following incubation, 0.1 ml of the enrichment broth No. 1 was placed onto one TSA plate (medium 3) and one EVTSA plate (medium 4) and spread over the plate with a glass rod. When duplicate samples were made, the second swab was treated as described above; except that it was placed into enrichment broth No. 2, which was

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[1]Difco Laboratories, Detroit, MI.

[2]National Veterinary Services Laboratories (NVSL), USDA, Ames, IA.

[3]Upjohn Co., Kalamazoo, MI.

[4]Baltimore Biologic Laboratory, Cockeysville, MD.

[5]Pfizer Diagnostic Division, N. Y., NY.

[6]Allide Chemical Co., N. Y., NY.

subcultured after incubation onto one TSA plate (medium 5) and one EVTSA plate (medium 6). Cultures were incubated at 37° C for 5-7 days.

Observation of Plates

The plates were examined by obliquely reflected light to estimate the number of brucella-like colonies after incubation. The colonies were counted and recorded by the following criterion; - = no growth, + = single colony, 2+ = 2-10 colonies, 3+ = 11-100 colonies, 4+ = 101-500 colonies, 5+ = greater than 500 colonies, C = plate where bacterial or fungal contaminants interfered with reading, and E = isolation by enrichment broth only.

Brucella-type colonies were gram-stained and subcultured to a TSA plate which was incubated at 37° C for 4-5 days. The plate was observed for purity and selected colonies were inoculated onto one potato agar slant with 5% bovine serum (PA)[1] and incubated at 37° C for 2-3 days.

After incubation, the PA slant containing growth of brucella-type colonies were stored at -20° C until needed for further identification tests.

Identification Tests

Brucella-type colonies from the plates were subjected to identification tests (Table 9) to differentiate strain 19 and strain 2308.

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[1]National Veterinary Services Laboratories (NVSL), USDA, Ames, IA.

Table 9. Growth-inhibition tests for differentiating B. abortus strain 19 and strain 2308

Tryptose agar medium containing	Strain 19	Strain 2308
Basic fuchsin[a]	+[b]	+
Thionin[a]	-[c]	-
Thionin blue[d]	-	+
Penicillin[e]	-	+
Erythritol[f]	-	+

[a]The following concentrations of dye were used; 1:25,000, 1:50,000, and 1:100,000.

[b]+ = growth.

[c]- = no growth observed.

[d]Dye concentration of 1:500,000.

[e]5 units/ml.

[f]1 mg/ml.

RESULTS

All isolates of B. abortus were confirmed to be either strain 19, the vaccine strain, or strain 2308, the challenge strain (Table 10). Brucella abortus isolates demonstrated characteristic growth patterns on differential media except strain 19 isolates from animal No. 30 which differed from the typical by consistent growth on erythritol medium.

Repeated recoveries of the same B. abortus strain were made from the samples collected during the shedding period of each individual animal. Isolation of both strains from an individual animal did not occur.

A total of 1770 vaginal samples were collected and cultured with the results shown in Table A7. Brucella abortus was isolated from the vaginal secretions of 7 of the 96 cows sampled (Nos. 20, 30, 62, 67, 69, 75, 82). The length of the shedding period as determined by positive cultures of B. abortus from each of the seven animals is shown in Figure 3. Strain 19 was isolated from two animals and strain 2308 from five animals. Animal No. 62 was the only one not challenged with strain 2308 due to abortion prior to the challenge date. The period of shedding of B. abortus from the vaginal secretions of the seven animals ranged from 2 to $10\frac{1}{2}$ weeks. Intermittent shedding was observed during the $4\frac{1}{2}$ week shedding period of animal No. 30 in which no positive cultures were made in the 3rd week.

Table 10. Growth inhibition test results for the characterization of B. abortus strain 19 and 2308 isolates from the vaginal mucus of seven cows.

Animal No.	No. of Isolates	B. abortus strain	Tryptose Agar Medium Containing				
			Basic Fuchsin[a]	Thionin[a]	Thionin[b] Blue	Penicillin[c]	Erythritol[d]
20	23	2308	+[e]	-[f]	+	+	+
30	8	19	+	-	-	-	+
62	3	19	+	-	-	-	-
67	32	2308	+	-	+	+	+
69	5	2308	+	-	+	+	+
75	5	2308	+	-	+	+	+
82	5	2308	+	-	+	+	+

[a]The following graded concentrations of dyes were added: 1:25,000, 1:50,000, and 1:100,000.

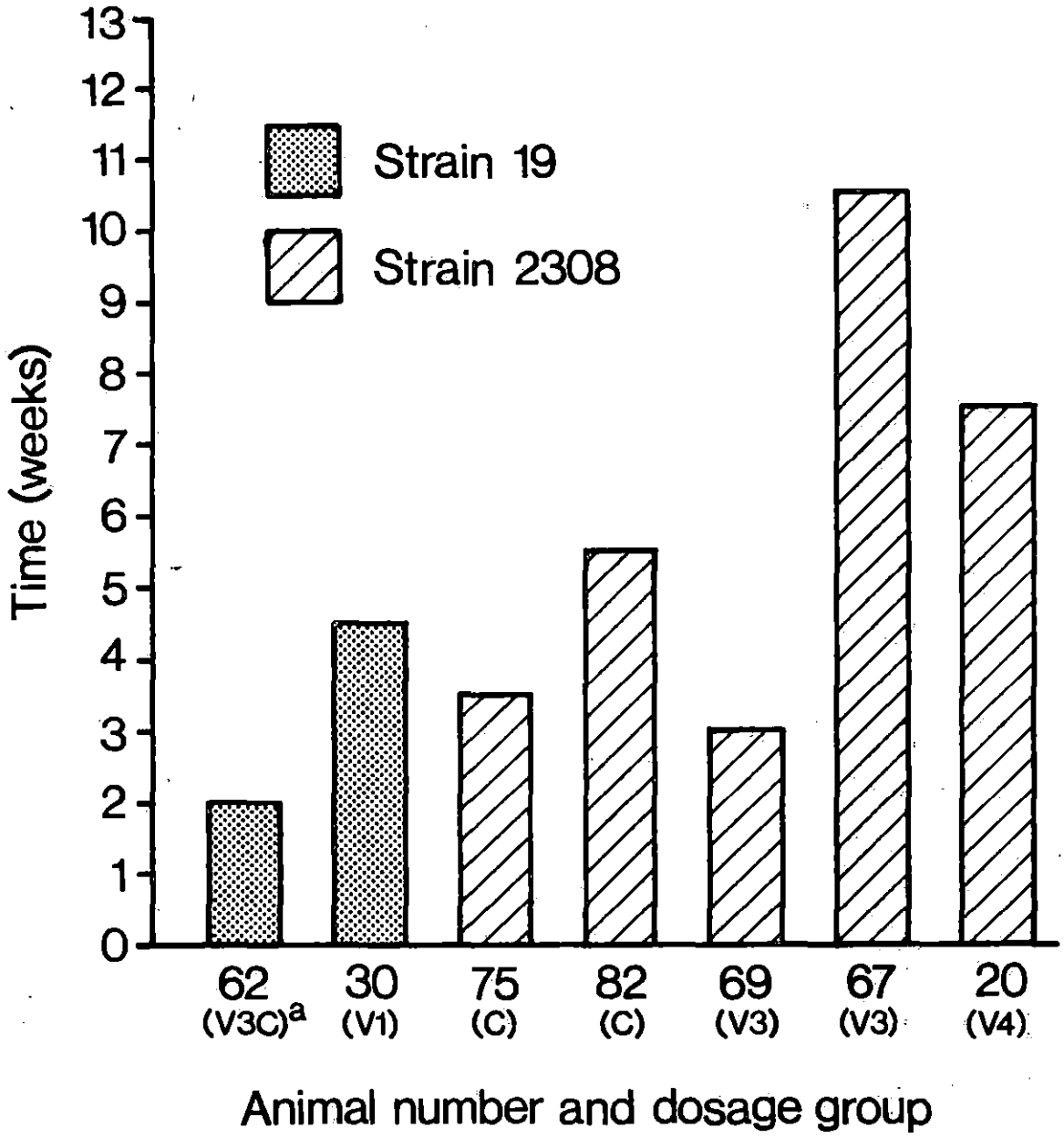
[b]Dye concentration of 1:500,000.

[c]5 units penicillin/ml.

[d]1 mg erythritol/ml.

[e]+ = growth.

[f]- = no growth.



[a]Animal not challenged with strain 2308.

Figure 3. Length of shedding period of B. abortus as detected in the vaginal mucus of seven cows post-challenge

No recoveries were made in a one week interval during the shedding period of animal No. 82, due to sample contamination.

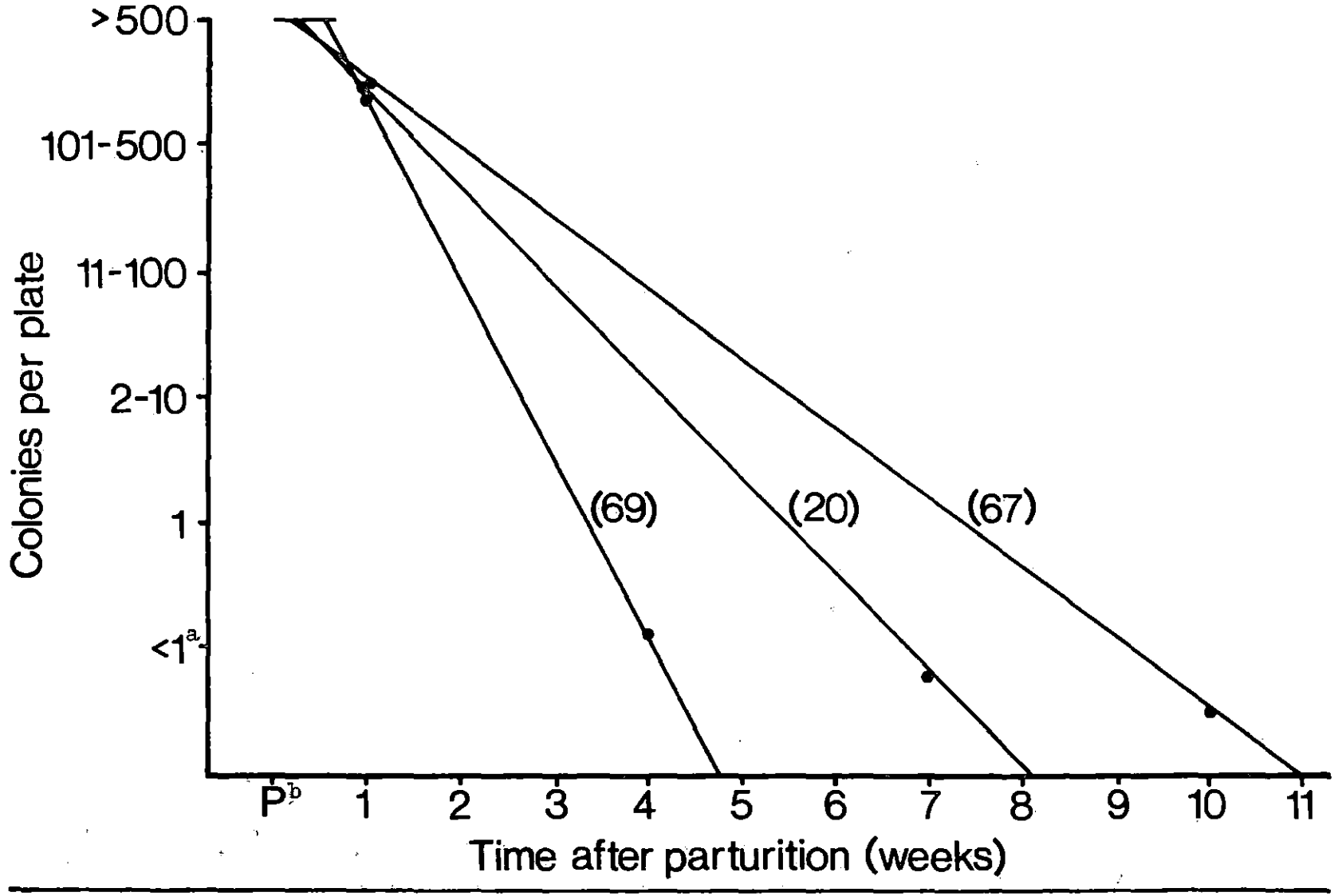
A similar trend of the various levels of shedding of B. abortus was detected throughout the isolation period for animal Nos. 20, 67, and 69 (Figure 4). Heavy shedding, more than 500 colonies per plate, was observed beginning at parturition and the number of organisms recovered appeared to decrease until isolations were no longer made.

The results of the number of positive cultures of B. abortus on solid media (TSA and EVTSA) by the direct streak plate method and in liquid medium by the enrichment broth technique are summarized in Table 11. Forty-six isolations of strain 2308 were made by the direct streak plate method, whereas 70 isolations of strain 2308 were made by the enrichment broth method. Eleven isolations of strain 19 were made by each of the methods. Therefore, a comparison of the total number of recoveries of strains 19 and 2308 revealed 57 were made by the direct streak plate method compared to 81 by the enrichment broth method. This finding is significant at p value = 0.005.

Contamination was observed in 62 of 1,770 (3.5%) vaginal samples cultured on TSA and EVTSA by the direct streak plate method compared to 165 or (9.3%) in the enrichment broth medium.

In three of the five animals from which strain 2308 was isolated, additional positive cultures were detected by the

Figure 4. The approximate numbers of colonies of B. abortus detected during the post challenge shedding period of animal Nos. 20, 67, and 69 (Data obtained from TABLE A8)



[a]Isolation by enrichment broth only.
[b]Parturition.

enrichment broth method compared to the direct streak plate method (Table 12). Recovery of strain 2308 was made for an additional two weeks by enrichment broth in cows No. 82 and 67, and for one additional week in cow No. 20. No isolations were made from the vaginal samples collected prior to parturition from any of the animals sampled by either method.

Detection of B. abortus by the enrichment broth method only was observed in 12 of the 29 duplicate samples collected at random from animals shedding B. abortus (Table 13). Five positive cultures were made only by the enrichment broth -1 containing SAS whereas 2 positive cultures were made only in the enrichment broth-2 containing no SAS.

Table 11. Number of positive cultures of Brucella abortus strains 19 and 2308 from the vaginal mucus as detected by the direct streak plate and enrichment broth methods

Brucella isolated	Direct streak plates	Enrichment broth	% Isolations Direct Streak plate / Enrichment broth
Strain 19	11	11	100
Strain 2308	46	70	65.7
Total	57	81	70.3

Table 12. Comparison of the number of B. abortus isolations from the vaginal mucus collected at weekly intervals following parturition of 5 cows challenged with B. abortus strain 2308 by two cultural methods

Animal No.	Method	No. weekly samples with isolations	Cultural results[a]
69	EB[b]	4	- + N + N + N + N - N - N -
	DSP[c]	4	
75	EB	4	- + N + N + N + N - N - N -
	DSP	4	
82	EB	6	- C + N + N C N + N ‡ N ‡ - - - - - -
	DSP	4	
20	EB	8	- + + + + + + ‡ + + + + + ‡ - ‡ - - - - -
	DSP	7	
67	EB	11	- + + N + + + + + + + + ‡ ‡ + N + N ‡ N + N ‡ N - - -
	DSP	9	

P[d] 1 2 3 4 5 6 7 8 9 10 11 12 13

Time after parturition (weeks)

[a] - = no isolation; + = isolation of strain 2308 from both methods; N = not sampled; C = contamination; ‡ = isolation of strain 2308 from enrichment broth only.

[b] Enrichment broth method.

[c] Direct streak plate method.

[d] P = parturition.

Table 13[a]. Comparison of the efficacy of enrichment broth with and without SAS for the isolation of B. abortus

I.	Samples examined - (29 duplicate)		58
II.	Total samples from which B. abortus recovered - (all methods)		50
	A. Recovered by direct plate[b]		38
	B. Recovered by enrichment broth only		12
	1. Enrichment broth-1 (SAS) only	5	
	2. Enrichment broth-2 (no SAS) only	2	
	3. Both enrichment broths, (SAS and no SAS)	5	

[a]Complete results listed in Table A9.

[b]Determined by the duplicate samples with the greater number of isolations observed by the direct streak plate method.

DISCUSSION

The enrichment broth technique increased by 29.7% the recovery rate of B. abortus from the vaginal mucus of cattle compared to the direct streak plate method (Table 12). Without the use of the enrichment broth, an additional 24 isolations would not have been made. The importance of the use of the enrichment broth technique was most clearly demonstrated when isolations were made from the vaginal mucus at the latter part of the shedding period when the number of organisms present were minimal (Table 13, Figure 4). Therefore, the use of only the direct streak plate method would have erroneously illustrated that shedding ceased at an earlier time. For example, animal No. 82 would have been reported to have shed for only 3.5 weeks rather than 5.5 weeks, and animals No. 20 and 67 would have been reported as intermittent shedders as well as for a shorter duration.

Bacteria other than B. abortus were in most cases effectively controlled by inhibitors incorporated in the enrichment broth. However, in some instances colonies of contaminants were more numerous on plates subcultured from the enrichment broth than on the direct streak plates. Similar observations were reported by Berkhoff in which contamination was 8.1% greater in a modified Brodie-Sinton fluid enrichment medium used for the isolation of B. abortus from milk than by direct plate culture (5). However, in Berkhoff's study, three additional isolations out of 42 positive cultures were made by the direct plate culture due to the contamination encountered in broth.

Philippon and Renoux encountered Proteus contamination on at least one culture plate made from the vaginal mucus sampled post-parturition in 60% of the cows examined (82). Overgrowth of cultures by non-brucella organisms was also observed in this study to be usually due to "swarmers".

The use of polyanions, SAS and SPS, as additives in blood culture media has been shown to improve the survival rate and increase the frequency of isolation of bacteria by inhibiting antimicrobial factors in serum (2, 23, 26, 37, 51, 54, 91, 92, 112). The addition of SAS to the tested selective broth appeared to have a potential advantage in increasing the recovery of B. abortus from the vaginal mucus of cattle (Table 13).

The mode of action of SAS is the inhibition of certain antimicrobial mechanisms of both natural and acquired immune systems (2, 4, 26, 37, 51, 53, 54, 91, 92, 111, 112). Complement-dependent phagocytosis has been shown to play an important role in engulfment of brucella in blood (111). The intracellular location of brucella in leukocytes may prevent their isolation. Therefore, the binding of complement by SAS may inhibit the phagocytosis of brucella in the vaginal mucus upon culture.

Studies by Payne demonstrated that brucella stimulate the production of cervicovaginal agglutinins in cattle (80). Other studies have shown that a high portion of the total protein in the vaginal secretions is due to serum components derived by transudation (16, 22, 62). SAS has an incomplete ability to inhibit serum agglutinins and may act in a similar

manner on cervicovaginal agglutinins. The inhibition by SAS of other antimicrobial proteins present in serum including lysozyme and B-lysins may also occur in the vaginal mucus.

The advantages of the use of SAS in blood culture media have been established. However, more information needs to be obtained before recommending its use in culture media used for the vaginal mucus or other body fluids.

The results obtained in this study pertaining to the length of shedding of B. abortus from the vaginal mucus in 4 of 7 cows (Figure 3) were similar to previous reports (3, 34, 64, 82, 83). Massive and continuous shedding was observed for about 2 weeks, followed by a decline which usually ceased in four to five weeks after parturition or abortion.

The trend (Figure 4) of the various levels of shedding of B. abortus throughout the isolation period was similar to the observations made by Philippon, Renoux, and Plommet (83). They reported that isolations became irregular after the first 17 days following parturition ceasing at about 5 weeks. However, in this study, B. abortus was isolated continuously except for a one week interval in one animal.

Additional samples were collected immediately prior to parturition and examined, but B. abortus was not recovered from any of these samples. Studies by Fitch et al. (34), and Manthei and Carter (64), did not detect brucella in the vaginal secretions of pregnant cows and concluded that shedding was linked to the freeing of the cervical plug. However, Philippon et al. demonstrated a minimal discontinuous shedding of B. abortus in the vaginal discharge starting the 39th day after challenge

(83). While the use of inadequate cultural procedures could be responsible for the lack of recovery of the organisms before parturition, the enrichment technique used in this study was demonstrated to be a sensitive method.

The differences observed in the isolation results of B. abortus prior to parturition compared to other studies may be related to the different experimental conditions including B. abortus vaccination and challenge strains used, and the vaccination schedule followed. Therefore, the immunity induced may have been different.

The shedding of B. abortus prior to parturition was studied by Philippon, Renoux, and Plommet and summarized by the following statement, "we noticed a certain correlation between the degree of brucellic infection of the carcass and the immediateness of vaginal elimination. Therefore, it will be permissible to believe in an early localization of B. abortus in the vaginal tissues, by the sanguine and lymphatic ducts (83)." Payne also supported the phenomenon of the early presence of B. abortus in the vaginal tissue on the basis that specific agglutinins in the vaginal discharge were observed before they appeared in the blood (80).

Injection of high dosages of strain 19 vaccine into pregnant cows has been shown to cause abortion followed by recovery of strain 19 from the genital tract (6, 38). Shedding of B. abortus in the vaginal mucus was detected in 7 of the 96 vaccinated animals of which 95 were also challenged. Five of the 95 challenged animals shed strain 2308. Strain 19 was isolated from two of the seven animals from which B. abortus was detected in the vaginal mucus.

The strain 19 isolates recovered from animal No. 62 which was vaccinated but not challenged had characteristics typical of USDA strain 19. A unique characteristic of the USDA strain 19, which differs from that of other B. abortus biotype-1 strains, is that it is unable to grow on a medium containing erythritol (Table A1). However, the organism repeatedly cultured from animal No. 30 (19/30), that was vaccinated and challenged, had characteristics different from those of either strain 19 or 2308. All 19/30 isolates were shown to have strain 19 characteristics, except that they were able to grow on medium containing erythritol. Strain 19 cultures obtained from Great Britian have the same characteristics as observed for 19/30 isolates and have been shown to have a comparable oxidation rate of erythritol as virulent strains of B. abortus (71).

Meyer reported that steroid hormones can induce filterable forms of B. abortus which exhibit altered characteristics after reversion (72, 73). It has also been reported that alteration in sensitivity to erythritol in B. abortus is the result of mutation (46). A limited study was conducted which subjected control cultures of strains 19 and 2308, and strain 19 isolates from animal Nos. 30 and 62 to various levels of progesterone used by Meyer. No alteration in the erythritol characteristic was observed in the cultures tested.

Another possibility is that genetic recombination between strains 19 and 2308 may have produced the 19/30 isolates. Further studies should be made to determine the cause of variation in erythritol sensitivity of 19/30 isolates.

SUMMARY AND CONCLUSIONS

1. A selective enrichment broth was developed for the isolation of B. abortus from the vaginal secretions of cattle. The medium was composed of tryptose broth to which hemin (5 ug/ml), bacitracin (25 units/ml), cycloheximide (100 ug/ml), nalidixic acid (5 ug/ml), polymyxin B sulfate (5 units/ml), vancomycin (25 ug/ml), and SAS (0.05%), were added.
2. The medium was then applied for comparisons of the patterns of shedding of B. abortus in adult vaccinated cattle. The shedding period of B. abortus from the vaginal secretions of seven animals ranged from 2 to 10½ weeks.
3. Eighty-one isolations of B. abortus were made from the 1770 samples collected, of which 70 isolates were characterized as the challenge strain 2308, and eleven isolates as the vaccination strain 19. However, in one instance, an atypical isolate, 19/30, was repeatedly cultured from animal No. 30, which had been vaccinated and challenged. All 19/30 isolates were shown to have strain 19 characteristics except that they consistently grew on erythritol medium.
4. The use of the selective enrichment broth proved to be more efficient (81 isolates) when compared to the direct streak method (57 isolates) ($p = 0.005$). Therefore, observations made in this study indicate that the enrichment broth method should be used in addition to direct streak plates when culturing vaginal secretions of cattle for field strains of B. abortus.

5. Although there were more contaminates encountered in the enrichment broth than the direct streak plate method, contamination did not interfere with isolation of B. abortus.

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APPENDIX

Table A1[a]. Differential characteristics of common biotypes and strains of Brucella abortus

Species	Biotype[b]	CO_2[c]	H_2S[d]	Growth on dyes	
				Thionin[e]	Basic fuchsin[e]
B. abortus	1	±[k]	+[l]	-[m]	+
	Strain 2308	-	+	-	+
	Strain 19	-	+	-	+
	2	+	+	-	-
	4	±	+	-	+

[a]Data processed previously described (1, 11).

[b]Those that occur in the United States.

[c]CO_2 requirement.

[d]H_2S production.

[e]Dye concentrations tested include 1:25,000, 1:50,000, 1:100,000.

[f]Dye concentration 1:500,000.

[g]1 mg/ml.

[h]5 units/ml.

[i]Abortus.

[j]Melitensis.

[k]± = variable characteristic for strains within the species.

[l]+ = possess characteristic.

[m]- = does not possess characteristic.

Growth on dyes	Growth on		Agglutination in monospecific serum		Phage lysis
Thionin blue[f]	Erythritol[g]	Penicillin[h]	A[i]	M[j]	at RTD
+	+	+	+	-	+
+	+	+	+	-	+
-	-	-	+	-	+
-	±	-	+	-	±
+	+	+	-	+	+

Table A2. Description of special medium preparations

Medium	Formula[a]	Source
Special tryptose broth (STB)	Beef extract - 3 gm	Difco[b]
	Sodium chloride - 5 gm	Fisher[c]
	Tryptose peptone - 10 gm	Difco
	Yeast extract - 5 gm	Difco
GC broth	Polypeptone peptone - 15 gm	BBL[d]
	Cornstarch - 1 gm	Argo[e]
	Dipotassium phosphate - 4 gm	Fisher
	Monopotassium phosphate - 1 gm	Fisher
	Sodium chloride - 5 gm	Fisher
	Hemoglobin - 2%	Difco
ANM broth	Proteose peptone No. 3 - 15 gm	Difco
	Cornstarch - 1 gm	Argo
	Dipotassium phosphate - 4 gm	Fisher
	Potassium dihydrogen phosphate - 1 gm	Fisher
	Sodium chloride - 5 gm	Fisher
	Sodium bicarbonate - 0.15 gm	Fisher
	Glucose - 5 gm	Fisher

[a]Each formula is described in grams per liter of distilled water. The pH was adjusted to 7.0 in all base media, and then autoclaved at 121° C for 15 minutes before inhibitory agents, hemin, and sodium amylosulfate solutions were added where indicated.

[b]Difco Laboratories, Detroit, MI.

[c]Fisher Scientific Co., Fairlawn, NJ.

[d]Baltimore Biological Laboratory, Cockeysville, MD.

[e]Argo, Best Foods, Englewood Cliffs, NJ.

Table A2. (Continued)

Medium	Formula	Source
Enrichment broth No. 1	Prepared tryptose broth - 26 gm	Difco
	Vancomycin - 25 ug/ml	Eli Lilly[f]
	Nalidixic acid - 5 ug/ml	Aldrich[g]
	Cyclohexamide - 100 ug/ml	Upjohn[h]
	Polymyxin B sulfate - 5 units/ml	Pfizer[i]
	Bacitracin - 25 ug/ml	Squibb[j]
	Hemin - 5 ug/ml	Eastman[k]
	Sodium amylosulfate - .05%	Searle
Enrichment broth No. 2	Prepared tryptose broth - 26 gm	Difco
	Vancomycin - 25 ug/ml	EliLily
	Nalidixic acid - 5 ug/ml	Aldrich
	Cyclohexamide - 100 ug/ml	Upjohn
	Polymyxin B sulfate - 5 units/ml	Pfizer
	Bacitracin - 25 ug/ml	Squibb
	Hemin - 5 ug/ml	Eastman

[f]Eli Lilly Co., Indianapolis, IN.

[g]Aldrich Chemical Co., Milwaukee, WI.

[h]Upjohn Co., Kalamazoo, MI.

[i]Squibb Pharmaceuticals, Princeton, NY.

[j]Eastman Kodak Chemical Co., Rochester, NY.

[k]Searle Laboratories, Chicago, IL.

Table A3. Growth responses of strain 19, strain 2308, Staph. aureus, and E. coli subjected to various concentrations of selected inhibitory agents

Antibiotic	Organism	Concentration of antibiotic[a]						Concentration recommended[b]
Amphotericin B (ug/ml)		40	20	10	5	2.5	C[c]	5
	Strain 19	+[d]	+	+	+	+	+	
	Strain 2308	+	+	+	+	+	+	
	Staph. aureus	+	+	+	+	+	+	
	E. coli	+	+	+	+	+	+	
Bacitracin (ug/ml)		80	40	20	10	5	C	25
	Strain 19	+	+	+	+	+	+	
	Strain 2308	+[e]	+	+	+	+	+	
	Staph. aureus	-	-	-	-	-	+	
	E. coli	+	+	+	+	+	+	
Cycloheximide (ug/ml)		250	125	62.5	31.25	15.6	C	100
	Strain 19	+	+	+	+	+	+	
	Strain 2308	+	+	+	+	+	+	
	Staph. aureus	+	+	+	+	+	+	
	E. coli	+	+	+	+	+	+	
Cycloserine (ug/ml)		1200	600	300	150	75	C	300
	Strain 19	-	-	+	+	+	+	
	Strain 2308	-	-	+	+	+	+	
	Staph. aureus	-	-	-	-	+	+	
	E. coli	-	-	-	+	+	+	
Nalidixic acid (ug/ml)		40	20	10	5	2.5	C	10
	Strain 19	+	+	+	+	+	+	
	Strain 2308	+	+	+	+	+	+	
	Staph. aureus	+	+	+	+	+	+	
	E. coli	-	-	-	-	-	+	

[a] Antibiotic concentrations were prepared in tryptose broth.

[b] Recommended concentrations described by (29).

[c] C = control.

[d] + = presence of growth.

[e] - = no growth.

Table A3. (Continued)

Antibiotic	Organism	Concentration of antibiotic						Concentration recommended
Nystatin (ug/ml)		140	70	35	17.53	8.8	C	100
	Strain 19	+	+	+	+	+	+	
	Strain 2308	+	+	+	+	+	+	
	Staph. aureus	+	+	+	+	+	+	
	E. coli	+	+	+	+	+	+	
Polymyxin B Sulfate (units/ml)		38.4	19.2	9.6	4.8	2.4	C	5
	Strain 19	-	-	-	+	+	+	
	Strain 2308	-	-	-	+	+	+	
	Staph. aureus	-	+	+	+	+	+	
	E. coli	-	-	-	-	+	+	
Vancomycin (ug/ml)		100	50	25	12.5	6.25	C	25
	Strain 19	+	+	+	+	+	+	
	Strain 2308	+	+	+	+	+	+	
	Staph. aureus	-	-	-	-	-	+	
	E. coli	+	+	+	+	+	+	

Table A4. Preliminary Trial 1 - Growth responses of strain 19 grown in various basic media, and media with selected additives

Liquid medium	Viable plate count (hours)			Growth index[a]
	0	12	24	24 hours
TB	8.5×10^5	4.7×10^7	11×10^7	129.4
STB	4.5×10^5	4.1×10^7	7.1×10^7	157.8
ANM	6.0×10^5	4.3×10^7	6.7×10^7	111.7
ANM-S	7.0×10^5	2.7×10^7	8.6×10^7	122.9
ANM-H-V	5.0×10^5	2.9×10^7	7.4×10^7	148.0
BB	8.0×10^5	$.7 \times 10^7$	7.0×10^7	87.5
BB-S	6.5×10^5	$.6 \times 10^7$	2.0×10^7	30.8
BB-H-V	10.6×10^5	1.65×10^7	5.0×10^7	70.8
BB-S-H-V	9×10^5	1.7×10^7	1.95×10^7	21.7
GC	9.5×10^5	3.4×10^7	14.5×10^7	152.6

[a]Growth index = $\frac{\text{viable colony count at 24 hours}}{\text{viable colony count at 0 hours}}$.

Table A5. Preliminary Trial 2 - Growth responses of strain 19 grown in various basic media, and media with selected additives

Liquid medium	Viable plate count (hours)			Growth index[a]
	0	12	24	24 hours
TB	7×10^5	7×10^7	8.5×10^7	121.4
TB-H	10×10^5	7.5×10^7	13.5×10^7	135.0
STB	13.5×10^5	6.6×10^7	14.0×10^7	103.7
STB-G	0×10^5	4.0×10^7	8×10^7	88.9
STB-G-H-K	10.5×10^5	6.0×10^7	12×10^7	114.3
ANM-H	10×10^5	8.5×10^7	11×10^7	110.0

[a]Growth index = $\frac{\text{viable colony count at 24 hours}}{\text{viable colony count at 0 hours}}$.

Table A6. Growth responses of strain 19 grown in tryptose broth containing different inhibitory agents, hemin, and SAS

Liquid medium	Viable plate count (hours)			Growth index (hours)[a]	
	0	12	24	12	24
TB	8×10^5	4.14×10^7	9.4×10^7	51.7	117.5
TB-1	8×10^5	5.05×10^7	9.5×10^7	63.2	118.8
TB-1-H	8.5×10^5	4.12×10^7	13.5×10^7	48.5	158.9
TB-2	8.5×10^5	1.92×10^7	9.6×10^7	22.6	113.0
TB-2-H	8.0×10^5	2.25×10^7	10.0×10^7	28.1	125.0
TB-2-H-SAS	10×10^5	4.4×10^7	11.5×10^7	44.0	115.0

[a]Growth index = $\frac{\text{viable colony count at 24 hours}}{\text{viable colony count at 0 hours}}$.

Table A7. Cultural results of all vaginal mucus samples collected from 96 cows challenged with strain 2308 and examined for B. abortus

Animal no.	Sampling dates	Medium[a]						Strain isolated
		Direct streak plate		Enrichment broth				
		1	2	3[b]	4[b]	5[c]	6[c]	
1	4-3 to 6-27, 7-7	-	-	-	-			
	6-30, 7-25	-	-	C	C			
	7-1	C	-	C	C			
	7-5, 7-11 to 7-18	C	C	C	C			
2	4-3 to 6-20, 7-1 to 8-22	-	-	-	-			
	6-27, 7-5	-	-	C	C			
3	4-3 to 7-11, 7-18 to 8-22	-	-	-	-			
	7-14	-	-	C	C			
4	4-3 to 8-29	-	-	-	-			
5	4-3 to 8-29	-	-	-	-			
6	4-3 to 7-14, 7-25, 7-28 to 8-22	-	-	-	-			
	7-28	-	-	C	C			
	7-18, 7-25	C	C	C	C			
7	4-3 to 6-27, 6-30, 7-11 7-25	C	-	C	C			
	6-29	C	-	C	C			
	7-5, 7-18	C	C	C	C			
8	4-3 to 7-25, 8-22	-	-	-	-			
	8-1, 8-8	C	C	C	C			
9	4-3 to 8-22	-	-	-	-			
10	4-3 to 5-23, 6-13, 6-27 to 8-22	-	-	-	-			
	5-30, 6-6, 6-20	-	-	C	C			
11	4-3 to 7-18, 7-25 to 8-22	-	-	-	-			
	7-2	-	-	C	C			

[a]1, 3, 5 = TSA agar plate; 2, 4, 6 = EVTSA agar plate; - = no isolation; C = contamination; + = 1 colony/plate; 2+ = 2-10 colonies/plate; 3+ = 11-100 colonies/plate; 4+ = 101-500 colonies/plate; 5+ = greater than 500 colonies/plate.

[b]Subculture of enrichment broth containing SAS.

[c]Subculture of enrichment broth not containing SAS.

Table A7. (Continued)

Animal no.	Sampling dates	Medium: Direct streak plate 1	Direct streak plate 2	Enrichment broth 3	Enrichment broth 4	Enrichment broth 5	Enrichment broth 6	Strain isolated
12	4-3 to 6-27, 7-5 to 7-25	-	-	-	-			
	7-3	-	-	C	C			
13	4-3 to 7-5, 7-18 to 8-22	-	-	-	-			
	7-11	-	-	C	C			
	7-7	C	C	C	C			
14	4-3 to 8-22	-	-	-	-			
15	4-3 to 7-25	-	-	-	-			
	8-22	C	C	C	C			
16	4-3 to 7-7, 7-11 to 7-28	-	-	-	-			
	7-8	-	-	C	C			
	8-17	C	C	C	C			
17	4-3 to 6-27, 7-7 to 8-22	-	-	-	-			
	7-5	-	-	C	C			
19	4-3 to 7-11, 7-21, 7-25	-	-	-	-			
	7-18	-	-	C	C			
20	4-3 to 6-30, 8-7, 8-9, 8-18, 8-22, 8-29 to 9-28	-	-	-	-			
	7-4, 7-5, 7-14, 7-18	5+	5+	5+	5+			2308
	7-11, 7-21	4+	4+	5+	5+			2308
	7-28	2+	2+	5+	5+			2308
	8-10, 8-14	+	2+	5+	5+			2308
	8-1, 8-4	+	-	5+	5+			2308
	8-8	-	+	5+	5+			2308
	7-25, 8-3, 8-11, 8-15, 8-24	-	-	5+	5+			2308
	8-4, 8-10, 8-11, 8-15, 8-18	-	-			5+	5+	2308
	8-1, 8-7, 8-9, 8-24 to 9-28	-	-			-	-	
21	4-3 to 6-27, 7-5 to 7-25	-	-	-	-			
	6-29	C	-	-	-			
	6-30	-	-	C	C			
22	4-3 to 7-5, 7-14 to 8-22	-	-	-	-			
	7-7	-	-	C	C			
	7-11	C	-	-	-			
23	4-3 to 6-27, 7-10 to 8-23	-	-	-	-			
	7-5	-	-	C	C			
	7-7	C	C	C	C			

Table A7. (Continued)

Animal no.	Sampling dates	Medium: Direct streak plate 1	Direct streak plate 2	Enrichment broth 3	Enrichment broth 4	Enrichment broth 5	Enrichment broth 6	Strain isolated
24	4-3 to 7-5, 7-18 to 7-25	-	-	-	-			
	7-11	-	-	C	C			
25	4-3 to 6-23, 7-11 to 7-25	-	-	-	-			
	6-27	C	C	C	C			
	6-30 to 7-5	-	-	C	C			
26	4-3 to 8-22	-	-	-	-			
27	4-3 to 6-29, 7-25	-	-	-	-			
	6-30 to 7-18	-	-	C	C			
	7-21	C	C	C	C			
28	4-3 to 7-5, 7-11 to 7-25, 8-22	-	-	-	-			
	8-1	-	-	C	C			
	7-7	C	C	C	C			
29	4-3 to 6-20, 7-5 to 8-22	-	-	-	-			
	6-27	-	-	C	C			
30	4-3 to 5-30, 6-13 to 6-30, 7-25 to 7-28, 8-15 to 9-5	-	-	-	-			
	6-6, 8-1	-	-	C	C			
	7-2	5+	5+	5+	5+			19
	7-11, 7-14	4+	4+	5+	5+			19
	7-5, 7-8	3+	3+	5+	5+			19
	7-21	3+	2+	5+	5+			19
	7-21	-	2+			5+	5+	19
	8-1	+	-			5+	5+	19
	8-8, 6-6	C	C	C	C			
31	4-3 to 6-6, 6-20 to 7-5, 7-11 to 7-25	-	-	-	-			
	6-13, 7-6	-	-	C	C			
32	4-3 to 6-20, 7-5 to 7-25	-	-	-	-			
	6-27	-	-	C	C			
33	4-3 to 8-22	-	-	-	-			
34	4-3 to 7-5, 7-14, 7-18, 8-22	-	-	-	-			
	7-17, 7-25, 8-1	C	C	C	C			
	7-11	-	-	C	C			

Table A7. (Continued)

Animal no.	Sampling dates	Medium						Strain isolated
		Direct streak plate		Enrichment broth				
		1	2	3	4	5	6	
35	4-3 to 7-5, 7-11 to 8-22	-	-	-	-			
	7-7	C	C	C	C			
36	4-3 to 7-11, 8-22	-	-	-	-			
	7-12	-	-	C	C			
	7-18, 7-25	C	C	C	C			
37	4-3 to 6-13, 6-27 to 7-14, 7-25	-	-	-	-			
	6-20, 7-18	-	-	C	C			
38	4-3 to 6-13, 6-27 to 8-22	-	-	-	-			
	6-20	-	-	C	C			
39	4-3 to 7-7, 7-25	-	-	-	-			
	7-11, 7-18	-	-	C	C			
40	4-3 to 8-22	-	-	-	-			
41	4-3 to 6-13, 6-27 to 7-5, 7-14 to 8-1	-	-	-	-			
	6-20, 7-11	-	-	C	C			
42	4-3 to 4-11, 5-9 to 6-27, 7-11, 7-18	-	-	-	-			
	4-17, 7-5, 7-25	-	-	C	C			
43	4-3 to 5-23, 6-6 to 6-20, 7-5, 7-11 to 7-25	-	-	-	-			
	5-30, 6-27, 7-6	-	-	C	C			
44	4-3 to 6-29, 7-5 to 7-25	-	-	-	-			
	6-30	-	-	C	C			
45	4-3 to 7-25	-	-	-	-			
46	4-3 to 6-13, 6-27, 6-30, 7-11 to 7-25	-	-	-	-			
	6-20, 6-29, 7-5	-	-	C	C			
47	4-3 to 6-20, 7-5 to 7-10, 8-22	-	-	-	-			
	6-27, 7-25	-	-	C	C			
	7-11, 7-18	C	C	C	C			
48	4-3 to 6-13, 6-27 to 8-22	-	-	-	-			
	6-20	-	-	C	C			
49	4-3 to 6-27, 7-5, 7-14 to 7-25	-	-	-	-			
	7-1 to 7-11	-	-	C	C			

Table A7. (Continued)

Animal no.	Sampling dates	Medium						Strain isolated
		Direct streak plate		Enrichment broth				
		1	2	3	4	5	6	
50	4-3 to 6-27	-	-	-	-			
	6-30 to 7-5, 7-18 to 7-25	-	-	C	C			
	7-11	C	C	C	C			
52	4-3 to 7-10, 7-25 to 8-22	-	-	-	-			
	7-18	-	-	C	C			
	7-11	C	C	C	C			
53	4-3 to 7-7, 7-14 to 7-17, 7-25 to 8-22	-	-	-	-			
	7-11	-	-	C	C			
	7-18	C	C	C	C			
54	4-3 to 7-21, 7-28 to 8-22	-	-	-	-			
	7-25	-	-	C	C			
55	4-3 to 7-5, 7-17, 8-1 to 8-22	-	-	-	-			
	7-11, 7-14, 7-18	-	-	C	C			
	7-25	C	C	C	C			
56	4-3 to 7-11, 8-22	-	-	-	-			
	7-25	-	-	C	C			
	7-14, 7-18	C	C	C	C			
57	4-3 to 7-25	-	-	-	-			
58	4-3 to 8-29	-	-	-	-			
60	4-3 to 7-25	-	-	-	-			
61	4-3 to 6-20	-	-	-	-			
	6-23 to 7-25	C	C	C	C			
62[d]	4-3 to 5-9	-	-	-	-			
	5-13, 5-23, 6-6, 6-20	5+	5+	5+	5+			19
	5-16	4+	4+	5+	5+			19
	5-30, 6-13, 6-27	-	-	C	C			
63	4-3 to 8-22	-	-	-	-			
64	4-3 to 7-11	-	-	-	-			
	7-18, 7-25	-	-	C	C			
64	4-3 to 7-11	-	-	-	-			
	7-18, 7-25	-	-	C	C			
65	4-3 to 8-22	-	-	-	-			

[d]Not challenged with strain 2308.

Table A7. (Continued)

Animal no.	Sampling dates	Medium: Direct streak plate 1	Direct streak plate 2	Enrichment broth 3	Enrichment broth 4	Enrichment broth 5	Enrichment broth 6	Strain isolated
66	4-3 to 6-13, 6-27 to 8-22	-	-	-	-			
	6-20	-	-	C	C			
67	4-3 to 7-7, 9-8, 9-28	-	-	-	-			
	8-16, 8-22, 8-24, 9-5	-	-			-	-	
	7-8 to 7-21	5+	5+	5+	5+			2308
	7-25, 8-9	4+	4+	5+	5+			2308
	7-27 to 8-3, 8-14	3+	3+	5+	5+			2308
	8-29	3+	2+	5+	5+			2308
	8-4 to 8-8, 8-25	2+	2+	5+	5+			2308
	8-10	2+	-	5+	5+			2308
	8-11, 9-12	+	+	5+	5+			2308
	8-15, 8-24	+	-	5+	5+			2308
	8-16, 8-18, 8-22, 9-5, 9-19	-	-	5+	5+			2308
	8-15, 8-18, 8-25, 8-29, 9-12, 9-19	-	-			5+	5+	2308
69	4-3 to 5-23, 6-23 to 7-11, 7-18 to 8-1	-	-	-	-			
	5-29, 5-30	5+	5+	5+	5+			2308
	6-6	4+	4+	5+	5+			2308
	6-13	3+	3+	5+	5+			2308
	6-20	2+	-	5+	5+			2308
	7-13	C	C	C	C			
70	4-3 to 8-22	-	-	-	-			
71	4-3 to 6-27, 7-9 to 8-22	-	-	-	-			
	7-5, 7-7	-	-	C	C			
72	4-3 to 5-23, 6-6 to 6-27, 7-18 to 7-25	-	-	-	-			
	5-30, 7-5	-	-	C	C			
	6-29, 6-30, 7-11	C	C	C	C			
73	4-3 to 8-22	-	-	-	-			
74	4-3 to 7-12, 8-22	-	-	-	-			
	7-18	-	-	C	C			
	7-25	C	C	C	C			
75	4-3 to 5-16, 6-20 to 6-27, 7-25 to 8-1	-	-	-	-			
	5-22 to 5-30	5+	5+	5+	5+			2308
	6-6	3+	4+	5+	5+			2308

Table A7. (Continued)

Animal no.	Sampling dates	Medium						Strain isolated
		Direct streak plate		Enrichment broth				
		1	2	3	4	5	6	
75	6-13	2+	-	5+	5+			2308
	7-5, 7-11	-	-	C	C			
	7-18	C	C	C	C			
76	4-3 to 8-22	-	-	-	-			
77	4-3 to 7-5, 7-11, 7-12, 8-22	-	-	-	-			
	7-7	-	-	C	C			
	7-18, 7-25	C	C	C	C			
78	4-3 to 7-5, 7-11 to 8-22	-	-	-	-			
	7-7	-	-	C	C			
79	4-3 to 8-22	-	-	-	-			
80	4-3 to 7-25	-	-	-	-			
81	4-3 to 8-22	-	-	-	-			
82	4-3 to 6-13, 7-18, 7-25, 7-28 to 8-29	-	-	-	-			
	7-21	-	-			-	-	
	6-20 to 6-27	5+	5+	5+	5+			2308
	7-11	3+	3+	5+	5+			2308
	7-21	-	-	5+	5+			2308
	7-25	-	-			5+	5+	2308
	6-18, 7-5	C	C	C	C			
83	4-3 to 6-27, 7-5 to 7-25	-	-	-	-			
	6-30	C	-	C	C			
84	4-3 to 8-1	-	-	-	-			
	8-22	C	C	C	C			
85	4-3 to 7-25, 7-31, 8-15 to 8-22	-	-	-	-			
	8-1	-	-	C	C			
	7-28	C	C	C	C			
86	4-3 to 8-22	-	-	-	-			
87	4-3 to 6-30, 7-11 to 7-31, 8-15 to 8-22	-	-	-	-			
	7-5, 8-1	-	-	C	C			
88	4-3 to 7-5, 7-8 to 7-25	-	-	-	-			
	7-7	-	-	C	C			

Table A7. (Continued)

Animal no.	Sampling dates	Medium: Direct streak plate 1	Direct streak plate 2	Enrichment broth 3	Enrichment broth 4	Enrichment broth 5	Enrichment broth 6	Strain isolated
89	4-3 to 6-27, 7-11, 7-18 8-1 to 8-22	-	-	-	-			
	7-7	-	-	C	C			
	7-5, 7-14, 7-25	C	C	C	C			
90	4-3 to 7-25	-	-	-	-			
91	4-3 to 6-27, 7-5, 7-7, 7-18 to 7-25	-	-	-	-			
	7-1, 7-11	-	-	C	C			
92	4-3 to 7-21, 7-27 to 8-22	-	-	-	-			
	7-25	-	-	C	C			
93	4-3 to 7-5, 7-16, 8-1 to 8-22	-	-	-	-			
	7-11, 7-18	-	-	C	C			
	7-14, 7-25	C	C	C	C			
94	4-3 to 8-22	-	-	-	-			
95	4-3 to 6-30, 7-18	-	-	-	-			
	7-5, 7-11, 7-25	-	-	C	C			
106	4-3 to 7-18	-	-	-	-			
	7-25	-	-	C	C			
116	4-3 to 7-5, 7-11 to 8-22	-	-	-	-			
	7-7	-	-	C	C			
119	4-3 to 8-22	-	-	-	-			
124	4-3 to 7-5, 7-21	-	-	-	-			
	7-7, 7-9	-	-	C	C			
	7-11 to 8-1	C	C	C	C			

Table A8. The approximate numbers of colonies of B. abortus strain 2308 detected during the post challenge shedding period of animals No. 20, 67, and 69

Time (weeks)	No. colonies/plate[a]		
	No. 20	No. 67	No. 69
P[b]	5+	5+	5+
.25	5+	N	
.75	N	5+	5+
1.25	4+	5+	4+
1.75	5+	5+	5+
2.25	5+	4+	5+
2.75	4+	3+	4+
3.25	< 1	3+	< 1
3.75	2+	3+	2+
4.25	1+	3+	1+
4.75	1+	2+	1+
5.25	1+	2+	1+
5.75	1+	< 1	1+
6.25	< 1	1	1+
6.75	N	< 1+	< 1
7.25	< 1	3+	< 1
7.75	0	N	0
8.25		< 1	
8.75		0	
9.5		1+	
10.5		< 1	
11.5		0	

[a] 5+ = greater than 500 colonies/plate, 4+ = 101-500 colonies/plate, 3+ = 11-100 colonies/plate, 2+ = 2-10 colonies/plate, 1+ = 1 colony/plate, < 1 = isolation by enrichment broth only, 0 = no isolation, N = not sampled.

[b] Parturition.

Table A9. Results of B. abortus isolation by enrichment broths containing SAS and without SAS from 29 duplicate sets of vaginal samples

Date	Animal No.	Media[a]					
		1	2	3	4	5	6
7-21	30	3+	2+	5+	5+		
		-	2+			5+	5+
7-21	82	-	-			-	-
		-	-	3+	3+		
7-25	20	-	-	5+	5+		
		-	-			5+	5+
7-25	82	-	-	-	-		
		-	-			5+	5+
7-27	20	-	2+			5+	5+
		-	2+	5+	5+		
7-28	20	2+	2+	5+	5+		
		2+	-			5+	5+
8-1	20	+	-	5+	5+		
		-	-			-	-
8-3	20	+	-	5+	5+		
		-	-			5+	5+
8-4	20	+	-	5+	5+		
		-	-			5+	5+
8-8	20	-	+	5+	5+		
		+	-			5+	5+
8-8	67	2+	2+	5+	5+		
		-	2+			5+	5+
8-10	20	+	2+	5+	5+		
		-	-			5+	5+
8-10	67	2+	-	5+	5+		
		2+	2+			5+	5+
8-11	67	2+	+			5+	5+
		+	+	5+	5+		
8-11	20	-	-			5+	5+
		-	-	5+	5+		
8-14	20	+	-			5+	5+
		+	2+	5+	5+		

[a]As previously described in Part II; 5+ = greater than 500 colonies/plate, 4+ = 101-500 colonies/plate, 3+ = 11-100 colonies/plate, 2+ = 2-10 colonies/plate, + = 1 colony/plate, - = no growth.

Table A9. (Continued)

Date	Animal No.	Media					
		1	2	3	4	5	6
8-15	20	-	-	5+	5+		
		-	-			5+	5+
8-15	67	+	-	5+	5+		
		-	-			5+	5+
8-18	20	-	-			5+	5+
		-	-	-	-		
8-16	67	-	-			-	-
		-	-	5+	5+		
8-18	67	-	-			5+	5+
		-	-	5+	5+		
8-22	67	-	-			-	-
		-	-	5+	5+		
8-24	20	-	-			-	-
		-	-	5+	5+		
8-24	67	-	-			-	-
		+	-	5+	5+		
8-25	67	2+	2+	5+	5+		
		-	-			5+	5+
8-29	67	3+	2+	5+	5+		
		-	-			5+	5+
9-5	67	-	-			-	-
		-	-	5+	5+		
9-12	67	-	+	5+	5+		
		-	-	5+	5+		
9-19	67	-	-	5+	5+		
		-	-			5+	5+