Development of filtration and comparison to other procedures for the isolation of <u>Brucella abortus</u> from bovine milk

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	5
Isolation of Brucella abortus from Milk	5
A historical perspective	5
Procedures and media for brucella isolation	8
Application of milk culture procedures	13
Thermal investigations of brucella viability	15
MATERIALS AND METHODS	17
Development of the Filtration Procedure	17
Cultures, media, and growth conditions	18
Bovine milk	18
Statistical analysis	19
Effect of surfactant on brucella viability	19
Effect of temperature and duration of exposure on brucella viability	20
Effect of trypsin on brucella viability	20
Effect of combined treatments on brucella viability	21
Direct Comparison of Four Isolation Procedures	22
Bovine milk samples	22
Isolation procedures	23
RESULTS	25
DISCUSSION	38
LITERATURE CITED	47
ACKNOWLEDGEMENTS	55

INTRODUCTION

Bovine brucellosis is a disease of concern not only to veterinary medicine, dairy, and beef industries but also to the public health. Brucellosis causes abortions, infertility, and decreased milk production in cattle, whereas, in humans, a chronic, debilitating, undulating, febrile illness is observed. The transmission of Brucella abortus (B. abortus) is through direct or indirect contact with an infected animal usually by consumption of infected dairy products, skin contact with infected tissue, and occasionally from aerosol exposure to laboratory and abattoir workers[88]. Estimated dairy and beef production losses due to brucellosis for 1983 exceeded $32 million in the United States (Personal communication: Dr. J. D. Huber, Cattle Diseases and Surveillance Staff, U.S. Dept. of Agriculture). The Federal Brucellosis Eradication Program has decreased the impact of this disease on the cattle industry. Estimated United States production losses for dairy in 1951 totaled $314 million (1981 dollars) (Personal communication: Dr. J. D. Huber). The program has striven to identify and eliminate infected animals to prevent the spread of the disease[64]. The diagnosis of brucellosis involves two approaches: serological tests and the culture of B. abortus from the infected animal.

Serological tests for antibodies to B. abortus are widely used and generally reliable but have their limitations. False positive serologic tests from cross-reactions with other bacteria due to shared antigens have

been observed (Francisella tularensis, Escherichia coli serogroup 0:157, Salmonella urbana, Pseudomonas maltophilia, Vibrio cholerae, and Yersinia enterocolitica serovar 0:9)[2]. Interpretation of serologic tests is sometimes difficult in the case of vaccinated animals because antibodies to the vaccine (B. abortus strain 19) cannot be distinguished from those stimulated by natural infection.

Another limitation is the isolation of B. abortus from serologically-negative animals in the early stages of infection. Since serological tests are more rapid than bacteriological culturing, they can identify potential candidates for culturing. A sensitive culture procedure would be helpful to resolve the diagnosis of brucellosis in problem herds with conflicting serological results.

Successful isolation of B. abortus is a definitive test for brucellosis. For the lactating animal, culturing of milk is advantageous over culture of tissue because it does not require that the animal be sacrificed. Culture of tissue is a one-time sampling compared to the possibility of obtaining multiple milk samples. Isolation of B. abortus from the milk of an infected cow may fail due to the intermittent shedding or shedding organisms in very small numbers. Isolation attempts on bovine milk from serologic reactor animals have consistently lower success rates than isolation from serologic reactor animal tissue. Isolation rates from bovine milk frequently range from 30 to 40 percent, whereas isolation rates from tissue range from 40 to 50 percent[45]. Although isolation rates are lower, the convenience of multiple sampling makes isolation attempts on bovine milk worthwhile. If the isolation rate for the culture of

bovine milk could be improved over the rate utilizing conventional centrifugation and selective media, milk culture would not only be convenient but supply additional epidemiological information.

The conventional centrifugation procedure for the isolation of B. abortus from bovine milk usually involves relatively large amounts of cream and milk solids containing intracellular brucella organisms in the somatic cells spread over selective media. It is possible that the cream and milk solids prevent contact of the bacteria with the surface of the media. If the cream and milk solids could be removed without decreasing brucella viability, and the brucella organisms could be concentrated, there would be an increased probability for isolating B. abortus.

A technique has been developed[78,12] for the direct enumeration of bacteria in raw milk after capture on a membrane filter. The entrapment of bacteria on a filter followed by their cultural recovery on appropriate media was thus suggested for the isolation of B. abortus from infected bovine milk. In order to pass the milk through a filter, treatment is necessary. Prefiltration treatment of the milk consists of digesting the milk protein, casein with trypsin and dispersing the milk fat globules with a surfactant plus heating.

The adaptation of this technique for the isolation of B. abortus from bovine milk has several aspects requiring investigation. The enumeration technique filtered only 2.0 ml of milk but larger amounts of milk must be filtered for comparison to the conventional brucella isolation procedure. If the prefiltration treatments would not affect brucella viability, a selective broth would recover organisms from the membrane filter. Using

such a procedure for the isolation of *B. abortus* might increase the success rate. The purpose of this investigation is to develop a filtration procedure for the isolation of *B. abortus* from bovine milk by determining the parameters for prefiltration treatments and to compare the filtration procedure with centrifugation, swabbing, and plating isolation procedures for naturally-infected milk.

LITERATURE REVIEW

Isolation of *Brucella abortus* from Milk

A historical perspective

The history of Brucellosis is a story having many threads woven into a tapestry of reality. Some of the threads were put into place by meticulous observation while other threads were pure serendipity. Some threads of observation were remarkably visionary for their time, having to wait to gain acceptance. Other threads were unraveled because they could not be verified. There were many contributors to the weaving of this tapestry, each adding their thread as they reported observations on what is now known as brucellosis.

Although there are several descriptions from ancient sources concerning a febrile illness called by several names (intermittent typhoid, remittent fever, Mediterranean fever, Undulant fever, and Malta fever), the first report of the causative agent was made by Sir David Bruce in 1887. At the time, he was a British medical officer detailed to the military and naval bases on the island of Malta in the Mediterranean Sea. There was a large population of civilian and military personnel on this small island who were experiencing a devastating febrile illness sometimes resulting in death. Sir David Bruce was able to isolate and describe the causative agent as *Micrococcus melitensis* by inoculating infected spleens into peptone-beef agar medium[16,17]. He utilized the name melitensis from the Malta island and identified the disease as

Mediterranean Fever. Sir David Bruce used the isolated *Micrococcus melitensis* to infect monkeys and recover the organism from them[18]. Although Sir David Bruce had reported the causative agent, the pathogenesis and epidemiology of this disease remained a mystery.

M. L. Hughes, an assistant of Bruce, wrote a monograph in 1897 that gave a detailed description of the disease he called Undulant Fever because of the recurrent febrile episodes of the patients[53]. In 1904, the Mediterranean Fever Commission was established to investigate measures to prevent this serious disease. While seeking to establish an animal model to study undulant fever, a local physician, T. Zammit, discovered the local goat population had strong serological reactions to the agglutination test[98]. He was able to isolate the causative organism from the goat's milk. Goats were the main source of milk for the island population. The morbidity and mortality of the military personnel decreased after they ceased consuming goat's milk, while the civilian population continued to suffer from the disease[33].

Another thread of the tapestry was contributed by Bang in 1897 when he isolated a bacterium from the infected uterus of a cow that had recently aborted. Bang named the bacillus-shaped organism which required a microaerophilic atmosphere for growth, abortion bacillus[5]. He recommended disinfection of the animal and the premises with segregation of infected animals from noninfected animals to minimize the spread of contagious abortion. Since investigators had not previously viewed abortion as infectious, these were novel recommendations and are still utilized. In 1906, Bang was able to infect a cow by ingestion of infectious material,

demonstrating the involvement of the alimentary tract in addition to the reproductive organs[6].

These two threads were not woven together for many years because of the different descriptions for bacterial shape, oxygen requirements, and affected-animal species. Alice Evans was the first to compare the two organisms (Micrococcus melitensis and Bacillus abortus), observing no differences[29]. Her work was verified by Meyer and Shaw[65] in 1920 and in addition they suggested the name of Brucella melitensis for the goat abortion organism and Brucella abortus for the cattle abortion organism to honor Sir David Bruce. In 1931, Huddleson[49] demonstrated several growth characteristics, such as oxygen requirement and sensitivity to dye medium, could differentiate Brucella abortus from the swine abortion organism, Brucella suis This work was confirmed by Kaber and MacLanahan in 1935[57].

There were several attempts in the early years to produce a vaccine. None had been devised that would stimulate an animal to produce a protective immunity but would not cause the clinical disease. Discovering this thread of brucellosis history was a combination of serendipity and meticulous observation. In 1923, Dr. Buck, working for the United States Department of Agriculture (U.S.D.A.), isolated several cultures from a Jersey cow named "Victor's Lady Matilda". These cultures remained on Dr. Buck's desk over 1 year before they were checked for virulence. The nineteenth culture demonstrated a stable, reduced, virulence. In 1936, this culture, Brucella abortus strain 19 was utilized as a live vaccine in field trials[44]. Not until 1941 was this vaccine approved and used in 39 states[14]. One difficulty with the use of a live vaccine was the confusion

between a virulent Brucella abortus isolate and the less virulent vaccine, Brucella abortus strain 19. Differential growth characteristics were established by Jones et al.[56] and later by Brown et al.[15]. When measured by these growth characteristics, strain 19 differed from the virulent challenge Brucella abortus strain 2308 in sensitivity to thionin blue and penicillin, utilization of erythritol, and CO_2 dependency. Strain 2308 was recovered in 1940 by Dr. Manthei of the U.S.D.A. from the aborted fetus of a cow which was in contact with cattle experimentally infected with a mixture of Brucella abortus cultures. It is not known if these various cultures were CO_2 dependent or independent[56].

Meyer and Cameron in 1961 demonstrated that each Brucella species had a characteristic and definitive pattern of oxygen uptake on selected amino acid and carbohydrate substrates[66,67]. This procedure is time consuming, expensive, and risks exposing laboratory personnel to brucella organisms, therefore has not replaced the conventional biotyping tests[94].

Brucella sp. classification tests have not changed over the years. Recent analyses by DNA restriction endonucleases have not been conclusive. Some reports indicate DNA homology between the Brucella species leads to the conclusion that there is only one species[95,46,71]. Other reports indicate DNA polymorphism concluding that the present classification based upon species and growth characteristics is acceptable[75,1].

Procedures and media for brucella isolation

The tapestry thread for the isolation of brucella was obscured by information on tubercle bacillus isolation. In 1894, the accepted

procedure for the isolation of tubercle bacilli from cattle was the inoculation of milk into guinea pigs[86]. Theobald Smith observed that some resultant lesions in the guinea pigs were not caused by the tubercle bacillus. Schroeder and Cotton in 1911 were able to isolate Bacillus abortus from the milk of an apparently healthy cow by inoculating guinea pigs[82]. This work was confirmed in 1913 by an assistant of Theobald Smith, M. Fabyan, who also described the pathogenesis in guinea pigs[35]. In 1913, Cotton reported on the length of time organisms were shed in cow's milk. He was able to isolate organisms 4.5 years after abortion and some cows that had never aborted were found to harbor organisms in their milk[22].

Evans, in 1915, was the first to isolate Bacillus abortus from bovine milk using a solid medium composed of lactose agar with 10% blood serum. In her bacteriological study of milk from infected cows, she noted brucella colony counts of herd milk and market milk that ranged from 19,000 to 20,000 per cc of milk. Evans asserted that the shape of the Bacillus abortus was coccoid, not that of a bacillus[28]. She observed that the cream is the source of the organisms. These data, combined with her 1918 study of Micrococcus melitensis and Bacillus abortus, prompted Evans to warn of the danger of the milk supply being contaminated with the abortus organism[29]. Her work was challenged by Theobald Smith who asserted that since no cases of human infection from the Bacillus abortus were reported, the milk was safe for consumption[87]. Isolation by guinea pig inoculation, requiring 3 to 4 weeks to recover the maximum number of organisms, was recommended by Smillie in 1918[84]. Cotton, in 1919, made several observations as to the location of the abortion organisms in

infected cattle. He stated that the uterus was a focal organ of infection only during pregnancy. Otherwise, the udder and the supramammary lymph nodes were the focal points of infection. Cotton was able to isolate organisms from the seminal vesicles but doubted the bull's involvement in dissemination of the disease. He reported that heating milk to 60°C for 15 minutes killed the organisms but heating it to 55°C for 20 minutes did not. Cotton recommended the pasteurization of milk, artificial insemination, separation of new additions to herds, and immunization with live vaccine[23]. The conflict of opinion concerning the safety of the public milk supply was not resolved for several years because of the inconsistent isolation of Bacillus abortus from milk[76].

Evans noted in 1919 that virulent strains of Bacterium abortus are not eliminated continuously in large numbers in the milk of cows which have aborted, even though the blood serum continues to react positively to the agglutination test[30]. Evans utilized the procedure of plating out the milk on solid medium, having results by one week. Other researchers preferred to inoculate guinea pigs because, though results took six to eight weeks, there was no contamination problem[38,97]. Some improvements were made in the solid media by Stafseth in 1920 by utilizing liver and spleen agar and incubation in a glass jar with the air exhausted by a suction pump[90]. Huddleson reported in the same year that the use of gentian violet in a liver agar medium controlled contamination[48].

As isolation methods were improved, reports[39] incriminated milk as a source of human infections. Pasteurization of all grades of milk replaced

regulations that separated the unpasteurized grade A milk from the other grades which were pasteurized. There was a plan to duplicate the tuberculosis regulations by requiring dairy cows to test negative for brucellosis and then keep their milk separate to prevent the spread of disease and thereby retain the perceived benefits of raw milk[89]. Huddleson et al. noted that other growth characteristics for the abortion organism including H_2S production and CO_2 requirement for isolation in a 1927 report[52].

Some investigators tried with limited success to establish a correlation between isolation and serological responses. Gilman recovered organisms from guinea pigs inoculated with milk in only 53.7% of the cases when the cows had agglutination titers of >1:80[42]. Bang and Bendixen observed that cows would continue to shed a range of a few to thousands of organisms for 1 or more lactations[7]. The guinea pig inoculation method was recommended by Henry et al.,[46] when the culture of centrifuged cream failed to isolate 20 to 30 percent compared to guinea pig inoculation. Isolation of B. abortus from milk with no agglutinating antibodies in the whey was observed by Gill in 1933[41]. Isolation of B. abortus from vaccinated and non-vaccinated cows with various serum agglutination titers was observed[19]. Thompson classified cows with serum titers of 1:80 to 1:500 having no history of abortions, as healthy carriers. He was able to isolate B. abortus from the milk of these healthy carriers[91]. Thompson also observed that B. abortus may localize in one quarter of the udder usually the right hind, and may be persistently shed throughout the entire lactation period provided mastitis does not intervene[92]. Dawson noted the

positive correlation between mastitis and brucella infection[27]. Sheldrake et al. observed the interaction of the mammary gland and intestinal lymph glands in production of antibodies to brucella organisms[83].

Two improvements of solid media for the isolation of B. abortus from bovine milk were the use of a complete peptone medium base by Ardrey in 1941[4] and the use of antibiotics to control contamination by Kuzdas and Morse in 1953[60]. A variety of antibiotics have been tested for brucella sensitivity and ability to control contamination[62,70]. Huddleson and White reported the sensitivity of B. abortus biotype 2 to crystal violet.[51] The unique udder lesions caused by this organism were observed by Huddleson[50]. Many formulations of media were tested for the growth of the biotype 2 organism[55,68,81,37].

Variations in the method of inoculating the solid media were attempted by Morgan and McDiarmid in 1960 by mixing the centrifuged cream and sediment prior to spreading it on to solid medium[69]. Nelson et al., in 1966, swabbed the cream and sediment on to 12 plates of media per sample. He examined 167 animals making 70 isolations of B. abortus originating from vaccinated animals and 65 isolations of B. abortus originating from non-vaccinated animals[72]. McCaughey and Hanna in 1969 used filtration of vaginal mucus to limit contamination prior to plating of the filtrate[63]. Brodie and Sinton in 1975 utilized a selective broth to supplement the plating of centrifuged cream and sediment. They noted an enhanced isolation rate of 10 to 18 percent by the fluid enrichment[13]. However, Berkhoff and Nicoletti, in 1978, concluded that the liquid medium was inferior to solid media for the culture of B. abortus from bovine milk[9].

The use of serum in the medium to counteract the toxic effects of amino acid imbalance, fatty acids, and elemental sulfur was the contribution of Gerhardt in 1958[40]. Bovine serum added to tryptose agar containing bacitracin, polymyxin B, cycloheximide, and ethyl violet was the medium of choice of Painter *et al*. in 1966[77]. They also suggested the same medium without the ethyl violet should be used simultaneously since *B. abortus* biotype 2 is sensitive to ethyl violet. Farrell utilized a serum dextrose basal medium with bacitracin, Vancomycin, Polymyxin B, nalidixic acid, nystatin, and cycloheximide in a study which resulted in 96% of the infected samples yielding brucella isolations, without inhibiting *B. abortus* biovar 2[36,80]. In 1977, Hunter and Kearns concluded that Farrell's medium was superior to other selective media for isolation and contamination control but grew smaller colonies[54]. Ewalt *et al*., in 1983, proposed a new selective medium (E medium) to replace the tryptose serum agar containing bacitracin, cycloheximide, and polymyxin B. The E medium is composed of tryptose agar to which bovine serum, bacitracin, cycloheximide, lincomycin, nystatin, and polymyxin B are added[32].

Application of milk culture procedures

The thread of knowledge derived from work done in culture procedures and media development is interwoven with other data in the evaluation of vaccines or epidemiological studies. Anderson *et al*., in 1962, emphasized the importance of cultural results in the interpretation of serological results in problem herds[3]. In a study in which animals were vaccinated followed by challenge, only sporadic recovery of *B. abortus* organisms was

made and no definite conclusion as to the efficacy of the vaccine was drawn[58]. Luchsinger et al., in 1973, commented on the urgent need to include bacteriologic culture procedures as an integral part of the epizootiological investigation used to confirm that brucellosis eradication has been achieved in a state or country[61]. Crawford et al., in a 1979 study of 4 Texas herds using adult vaccination, noted that the frequency of strain 19 isolation suggests that valuable information can be gained from biotype identification of B. abortus isolates from selected reactor cattle following adult vaccination[24]. Ewalt and Harrington, in 1979, reported cultural results on 104 cows that were serologically positive on the card test. Strain 19 was isolated from 22 animals, B. abortus biovar 1 was isolated from 9 animals, and 2 animals had a dual infection of strain 19 plus biovar 1[31]. Nicoletti, in 1981, isolated B. abortus from 744 of 1735 attempts as part of an adult vaccination study. Of the 744 isolates, 98 were identified as strain 19 and were related to the size of strain 19 dose and length of time after vaccination[73]. Huber and Nicoletti, in 1986, noted that adult vaccinated cattle yielded more brucella organisms per culture than those from non-vaccinated animals. He observed some animals that were serologically negative yet culture positive with strain 19[47]. Cordes made a similar observation in a study of 102 infected animals. Brucella isolations from serologically negative animals comprised 34.2% of the total number isolated[21]. Nielsen and Duncan, in 1988, concluded from a vaccination study that the isolation of organisms is important in the interpretation of the aberrant immune responses due to persistent vaccine strain infection[74].

The importance of B. abortus strain 19 infections due to an increased occurrence is indicated by comparing isolation results from national reference centers. Harrington and Bond, in 1977, indicated that strain 19 infections accounted for 8.9% of all tissue isolates and 1.7% of all milk isolates[45]. Crichton and Medveczky reported in 1987 that strain 19 constituted 22.2% of all isolates for the period 1981 through 1985. They observed that this increase in the number of strain 19 isolates was due to an increase in adult vaccination[25].

Thermal investigations of brucella viability

The thread of the brucella tapestry relating to isolation of brucella from milk is akin to the concerns of the safety of the milk supply for human consumption[79]. As the reports of brucella isolations mounted, the question of brucella viability in milk arose. Cotton, in 1919, noted that the abortion organism was killed when heated to 60°C for 15 minutes but survived at 55°C for 20 minutes[23]. Boak and Carpenter in 1928 studied 8 strains of Brucellae by heating to 140°F for 15 minutes and were not able to isolate any organisms, even when guinea pigs were inoculated with the heated milk[10]. In 1931, Boak and Carpenter noted that Brucella suis required the highest temperature to kill. For these cultures heating to 142 or 145°F for 20 or 30 minutes was sufficient to kill the brucella organisms[11]. New procedures for the pasteurization of milk required new thermal resistance studies. Smith, in 1932, was able to isolate brucella organisms from 9.4% of pasteurized commercial milk. Heating milk to 145°F

(63°C) for 30 minutes was sufficient to inactivate brucella organisms but heating to 145°F with zero holding time yielded 18.7% isolation of brucella organisms[85]. This work was confirmed by Gilman and Milks in 1933[43]. By extrapolating the thermal death time curve established for brucella, Kronenvett et al. in 1954 estimated the margin of safety to be 26 minutes for milk heated to 143°F (60°C) for 30 minutes. At higher temperatures and short time pasteurization of 161°F (71.7°C) for 15 seconds the margin of safety is 12 seconds[59]. This work was confirmed by Davies and Casey[26] plus Vanden Heever et al.[93]. Barrow et al. in a 1968 survey of pasteurized cream products noted that brucella organisms were isolated following flash heating at 85°C and 88°C in 19 out of 1,161 samples while heating to 70°C for 30 minutes was sufficient to prevent isolation of any brucella organisms[8]. Allowing the milk to sour rather than being pasteurized is not effective in killing brucella organisms[34]. Adherence to the present regulations for the pasteurization of milk will safeguard the public from potential infection with brucellosis.

MATERIALS AND METHODS

Development of the Filtration Procedure

Essential to application of the filtration procedure for the isolation of B. abortus from bovine milk was the feasibility of filtering 10 ml of milk and the effects of prefiltration treatments on brucella viability. The bacterial enumeration procedure utilized prefiltration treatments of: surfactant (Triton X-100, 1.0%), heat (50°C for 10 minutes), and trypsin (20% weight/volume). Since these treatments did not permit the filtration of larger volumes of bovine milk, several modifications were evaluated. In addition to Triton X-100 being used as a surfactant, Tween 80 (0.1% and 1.0%) was considered. The enzyme treatment was increased to two 30-minute treatments with 1 ml of 25% lyophilized trypsin. These modifications allowed the filtration of 10 ml bovine milk and identified the prefiltration treatments affecting brucella viability. Brucella viability was measured following prefiltration treatments by surfactant, effect of heat, trypsin, and the accumulative effect of these treatments. The measurements of brucella viability established the parameters of the filtration procedure. All work was done in a laminar flow class II biosafety cabinet and all procedures conformed to the accepted safety instructions for handling this pathogenic organism.

Cultures, media, and growth conditions

Cultures of *B. abortus* biovars 1, 2, 4, and strain 19 were provided by the Diagnostic Bacteriological Laboratory (DBL) of the National Veterinary Services Laboratories (NVSL). Cultures were recent isolations from infected cattle. The cultures were grown on tryptose agar with 5% bovine serum (TSA) for 2 days. Incubation for all brucella growth was 37° C in an atmosphere of air and 10% CO_2. The medium utilized in plating all the *B. abortus* milk suspensions was TSA with antibiotics (Bacitracin, Cycloheximide, and Polymyxin B) [TSAA]. Observation of brucella growth was facilitated by Henry's method[2] (7.5 x magnification with a dissecting microscope illuminated by substage light at a 45° angle). Representative colonies suspected of being brucella were subcultured on TSA medium and identified by conventional methods (dye tolerance, growth on penicillin and erythritol, Tb phage, urease, H_2S production, A and M antigen, CO_2 dependence and catalase)[2].

Bovine milk

Bovine milk used to prepare the suspensions of the 4 brucella cultures was obtained from a brucellosis-free herd. After separation by gravity, most of the cream was removed. The milk was then placed in a 69°C water bath for 30 minutes to destroy contaminating organisms.

Statistical analysis

Tests to determine the effects of prefiltration procedures on brucella viability were repeated three times and the data subjected to analysis of variance by calculating an F value. Treatments that caused significant differences in brucella viability were not utilized in the filtration process.

Effect of surfactant on brucella viability

Disk diffusion procedure was utilized to determine brucella viability after surfactant treatment. The brucella cells were suspended in sterile saline and 4 ten-fold dilutions were prepared in sterile saline. The diluted suspensions were inoculated onto TSA plates using cotton swabs to obtain lawns of growth.

Analytical paper disks (3 mm) were saturated with two concentrations of Tween 80 (0.1% and 1.0%) and Triton X-100 (0.1% and 1.0%). Each disk was placed onto a quadrant of the inoculated plates for each of the four *B. abortus* cultures. Following incubation for 2 days, plates were observed for inhibition of brucella growth. Zones of growth inhibition were measured on those plates on which the the highest dilution yielded confluent growth.

Brucella viability following surfactant treatment was also determined by a dilution plating method. After the four brucella cultures were suspended in 0.85% saline and mixed with milk, a 2 ml aliquot of the

brucella suspension was mixed with 2 ml of Tween 80 (0.1% or 1.0%). After a 10-minute exposure at 37°C, 1 ml of the treated suspension was spread onto TSAA. Colony counts made at 7 days were compared to non-treatment controls.

Effect of temperature and duration of exposure on brucella viability

The maximum treatment temperature and duration of exposure not effecting brucella viability were determined by the milk dilution procedure. Two ml aliquots of the four brucella milk suspensions were heated for 10 minutes in a water bath at 45°C, 50°C, and 55°C. Two ml aliquots of the four brucella suspensions were placed in the 50°C water bath for 20 minutes. Following heating, each suspension was divided and spread onto two plates of TSAA medium. One ml aliquots of the four brucella cultures suspended in milk were plated onto TSAA medium as controls. After 7 days, colony counts of the heated suspensions were compared to colony counts of unheated controls.

Effect of trypsin on brucella viability

Effects of trypsin on brucella viability was determined by a dilution plating method. Trypsin (1:250 Difco) was dissolved in sterile water at a concentration of 25% (weight/volume). After the trypsin solution was filter sterilized, 1 ml aliquots were placed in 20x125 mm test tubes and frozen at -20°C. Each of the 4 brucella cultures was exposed to trypsin by the addition of 1 ml of the 25% trypsin solution to 10 ml of the

brucella culture suspended in milk at 37°C. After 30 minutes, another 1 ml of 25% trypsin solution was added to the milk suspension and allowed to react for another 30 minutes at 37°C. The treated milk suspension was heated in a 50°C water bath for 10 minutes to inactivate the enzyme before plating 1 ml onto TSAA medium. Colony counts at 7 days were compared to colony counts of untreated milk suspensions.

Effects of combined treatments on brucella viability

The effects of combined treatments on *B. abortus* biovar 1 viability was determined by a plate dilution test. Milk suspensions of *B. abortus* biovar 1 were diluted to an estimated concentration of 100 organisms per ml. Ten ml aliquots of the milk suspensions were treated separately with heat, trypsin, or Tween 80. Individual milk suspensions were also treated with three combinations of the treatments: trypsin plus heat, trypsin plus Tween 80, or trypsin plus Tween 80 plus heat. One 10 ml aliquot was placed in to a 50°C water bath for 5 minutes prior to plating. One 10 ml aliquot was treated with 1 ml of 25% trypsin at 37°C for 30 minutes followed by an additional 1 ml of 25% trypsin at 37°C for 30 minutes prior to plating. One 10 ml aliquot was treated with 10 ml of 1.0% Tween 80 for 5 minutes prior to plating. One 10 ml aliquot was treated with two consecutive 30-minute treatments of 1 ml of 25% trypsin plus a 5-minute treatment in a 50°C water bath prior to plating. One 10 ml aliquot was treated with two consecutive 30-minute trypsin treatments plus 10 ml of 1.0% Tween 80 for 5 minutes prior to plating. One 10 ml aliquot was

treated with two consecutive 30-minute trypsin treatments plus 10 ml of 1.0% Tween 80 for 5 minutes plus a 5-minute treatment in a 50°C water bath prior to plating. After treatments, two 1 ml aliquots of the treated milk suspensions were plated onto TSAA plates. One ml of the untreated brucella milk suspension was plated onto TSAA plates as controls. Treatment colony counts at 7 days were compared to colony counts of non-treatment controls.

Direct Comparison of Four Isolation Procedures

Four procedures (swabbing, plating, centrifugation, and filtration) were evaluated on naturally infected milk for the isolation of B. abortus. The evaluation of the experimentally infected milk demonstrated the relative effectiveness of the four isolation procedures. Isolation rates for the different procedures were analyzed by the Cochran's Q-Test for correlated proportions.

Bovine milk samples

Naturally infected milk was obtained from submissions to the NVSL-DBL. These samples were received frozen, processed, and refrozen prior to the comparison of the four isolation procedures. A minimum of 22 ml per sample was utilized. If there was sufficient quantity, milk quarter samples were processed separately, otherwise the milk quarter samples from the same animal were combined.

Isolation procedures

Two TSAAEV plates were swabbed and two 1 ml aliquots of the milk sample were plated on to TSAAEV plates. A 10 ml aliquot of the milk quarter sample was centrifuged at 7700 x g for 15 minutes. The resultant cream was swabbed on to four different plates of media. (TSAA[77], TSAAEV[77], E medium[32], and Farrell's medium[37]). The skim milk was discarded into a disinfectant. The sediment was swabbed on to the four different plates of media (TSAA, TSAAEV, E medium, and Farrell's medium).

A 10 ml aliquot of the milk quarter sample was treated prior to filtration with two 30-minute treatments of 1 ml 25% trypsin solution at 37°C followed by the addition of 10 ml of 1.0% Tween 80 prior to a 5-minute heating in a 50°C water bath. This treatment digested the casein and dispersed the milk fat allowing the resultant liquid to pass by vacuum pressure through a series of membrane filters. The vacuum pump was located outside the laminar flow hood and had a 0.22 um in-line filter to prevent aerosol contamination of the pump. Cellulose nitrate filters (47 mm) and cellulose acetate filters (47 mm) having progressively smaller pore sizes (5.0, 1.2, 0.8, 0.06, and 0.45 um) were utilized in a clamped filtration apparatus. After filtration, the apparatus was rinsed with 1.0% Tween 80. The filtered milk was decanted into a clean container, 1 ml was plated on to a plate of TSAAEV for isolation of organisms escaping the filter, and the remainder was placed into the 50°C water bath while the filter was changed. If the initial cellulose nitrate filter became

clogged due to binding of proteins, a cellulose acetate filter of the same pore size would allow filtration on the sample. After filtration of the same pore size, filters were placed together in a 60 x 15 mm culture dish with 5 ml of the selective broth[13] and incubated. After 2 and 4 days, 1 ml of each pore size filter broth was plated on to one of the selective solid media.

Choice of the media to use was based on the anticipated level of contamination, number of days post-inoculation of the broth, and amount of antibiotics in the medium. The sequence of media to be chosen was first TSAA followed by E medium, TSAAEV, and Farrell's medium. The inoculated plates were observed for suspicious colonies after 1 and 2 days. If, in the recovery of the organisms from the filter broth, there was brucella growth in the rough form or excessive contamination, 10 ml of the Brodie's selective broth was inoculated with 0.5 ml of the filtration broth. Two ten-fold dilutions of the second broth were made. The second inoculated broth and the ten-fold dilutions were then incubated for 24 hours prior to plating on Farrell's medium. The inoculated plates were observed for suspicious colonies after 1 and 2 days. Suspicious colonies from each isolation procedure were confirmed by conventional methods at NVSL-DBL.

Quality control of the selective broth, 1.0% Tween 80, and filters was accomplished by plating 1 ml of the broth and 1 ml of the Tween 80 plus incubating the various filters used with separate 5 ml aliquots of broth prior to plating. The inoculated plates were incubated for 2 days before observing for bacterial growth.

RESULTS

The surfactant investigation is summarized in Tables 1 and 2. *Brucella abortus* biovar 2 viability was decreased by both concentrations of Triton X-100 in the disk diffusion test. The other brucella cultures were not inhibited. Neither concentration of Tween 80 had any effect on brucella viability as measured by the disk diffusion test. Milk suspensions of *B. abortus* biovar 1, biovar 2, biovar 4, and strain 19 had colony counts not significantly different ($p>0.90$, 0.80, 0.05, and 0.30 respectively) between the control and Tween 80 treatments of the plate dilution test.

The effects of temperature and the duration of heating on milk suspensions of the four *B. abortus* cultures are summarized in Tables 3-6. In Table 3, only the *B. abortus* strain 19 culture was significantly affected when heated at 45°C ($p<0.05$). In Table 4, the biovar 1 and the strain 19 *B. abortus* cultures were not significantly affected when treated at 50°C ($p>0.20$ and 0.30 respectively). The *B. abortus* biovar 2 and 4 cultures had viability decreased significantly when treated at 50°C ($p<0.01$). In Table 5, the 55°C treatment significantly affected the viability of *B. abortus* biovar 2 ($p<0.05$) plus biovar 4 and strain 19 ($p<0.01$). The *B. abortus* biovar 1 viability was reduced but not significantly affected ($p>0.10$). In Table 6, the 50°C treatment for 20 minutes significantly affected viability of *B. abortus* biovar 1 ($p<0.05$) plus biovar 2 and 4 ($p<0.01$). The *B. abortus* strain 19 viability was reduced but not significantly affected ($p>0.05$).

Table 1. Effect of surfactant on brucella viability measured by disk diffusion zones of inhibition[a]

Brucella cultures	Concentration of surfactant 0.1% Tween 80	1.0% Tween 80	0.1% Triton X-100	1.0% Triton X-100
B. abortus biovar 1	0	0	0	0
B. abortus biovar 2	0	0	16	31
B. abortus biovar 4	0	0	0	0
B. abortus strain 19	0	0	0	0

[a]Measured in mm.

Table 2. Effect of Tween 80 on brucella viability measured by plate dilution colony counts[a]

	Colony counts of Brucella abortus Biovar 1	Biovar 2	Biovar 4	Strain 19
Control	246	41	222	159
0.1% Tween 80	245	40	185	173
1.0% Tween 80	253	42	194	188
Analysis of variance	NS[b]($p>0.90$)	NS($p>0.80$)	NS($p>0.05$)	NS($p>0.30$)

[a]Colony counts were averages of 3 trials.

[b]NS = Not significantly different.

Table 3. Effect of 45°C heating for 10 minutes on brucella viability measured by plate dilution colony counts[a]

	Colony counts of Brucella abortus			
	Biovar 1	Biovar 2	Biovar 4	Strain 19
Control A	107	42	127	191
45°C - 10 minutes	98	36	138	122
Analysis of variance	NS[b]($p>0.60$)	NS($p>0.70$)	NS($p>.15$	S[c]($p<0.05$)

[a]Colony counts were averages of 3 trials.

[b]NS = Not significantly different.

[c]S = Significantly different.

Table 4. Effect of 50°C heating for 10 minutes on brucella viability measured by plate dilution colony counts[a]

	Colony counts of Brucella abortus			
	Biovar 1	Biovar 2	Biovar 4	Strain 19
Control A	71	21	205	55
50°C - 10 minute	92	8	33	61
Analysis of variance	NS[b]($p>0.20$)	S[c]($p<0.01$)	S($p<0.01$)	NS($p>0.30$)

[a]Colony counts were averages of 3 trials.

[b]NS = Not significantly different.

[c]S = Significantly different.

Table 5. Effects of 55°C heating for 10 minutes on brucella viability measured by plate dilution colony counts[a]

	Colony counts of Brucella abortus			
	Biovar 1	Biovar 2	Biovar 4	Strain 19
Control	71	21	205	55
55°C - 10 minutes	47	11	9	11
Analysis of variance	NS[b]($p>0.10$)	S[c]($p<0.05$)	S($p<0.01$)	S($p<0.01$)

[a]Colony counts were averages of 3 trials.

[b]NS = Not significantly different.

[c]S = Significantly different.

Table 6. Effect of 50°C heating for 20 minutes on brucella viability measured by plate dilution colony counts[a]

	Colony counts of Brucella abortus			
	Biovar 1	Biovar 2	Biovar 4	Strain 19
Control	71	21	205	55
50°C - 20 minutes	36	1	2	37
Analysis of variance	S[b]($p<0.05$)	S($p<0.01$)	S($p<0.01$)	NS[c]($p>0.05$)

[a]Colony counts were averages of 3 trials.

[b]S = Significantly different.

[c]NS = Not significantly different.

Trypsin prefiltration treatment effects on brucella milk suspensions are summarized in Table 7. Milk suspensions of B. abortus biovars 1, 2, and 4 had a significant decrease in brucella viability after trypsin prefiltration treatment ($p<0.05$). Viability of B. abortus strain 19 was reduced but not significantly affected ($p>0.30$).

Table 7. Effect of trypsin on brucella viability measured by plate dilution colony counts[a]

	Colony counts of Brucella abortus			
	Biovar 1	Biovar 2	Biovar 4	Strain 19
Control	307	279	354	69
Trypsin treatment	161	69	233	20
Analysis of variance	S[b]($p<0.05$)	S($p<0.05$)	S($p<0.05$)	NS[c]($p>0.30$)

[a]Colony counts were averages of 3 trials.

[b]S = Significantly different.

[c]NS = Not Significantly different.

The effect of combined prefiltration treatments on brucella milk suspensions as determined by the plate dilution test is summarized in Table 8. The colony counts for the Tween 80 treatment were adjusted for the volume disparity with the other treatments. The Tween 80 counts were doubled because of the dilution effect from treatment volume (10 ml of Tween 80 plus 10 ml of the milk suspension). B. abortus biovar 1 culture showed no significant loss of viability when control milk suspensions were compared to either single treatments or combinations of the treatments ($p>0.10$).

Table 8. Effect of combined treatments of Brucella abortus biovar 1 viability measured by plate dilution colony counts[a]

Treatment	Colony counts
Control	88
Heat 50°C - 5 minutes	90[b]
Trypsin	72
Trypsin + Heat	76
Trypsin + Tween 80	79
Trypsin + Tween 80 + Heat	84
Tween 80	77

[a]Colony counts were averages of 3 trials.

[b]All treatments not significantly different from control ($p>0.10$).

The number of _B_. _abortus_ isolations from naturally infected milk by 4 procedures is summarized in Table 9. Of the 75 isolation attempts, 26 were successful in isolating _B_. _abortus_ utilizing 1 or more of the procedures, but 49 attempts failed to isolate _B_. _abortus_ utilizing any of procedures. Eleven samples yielded _B_. _abortus_ by all four of the isolation procedures. Four isolations were made by swabbing, centrifugation, and filtration but not plating. Three isolations were observed by the filtration procedure only. Two isolations were made by the filtration and centrifugation but not swabbing or plating and two by the centrifugation and swabbing procedures but not the plating or filtration procedures. One sample was positive by the centrifugation, swabbing, and plating procedures but not by the filtration procedure and one sample by the plating, centrifugation, and filtration procedure but not the swabbing procedure. One sample yielded a brucella colony by the plating procedure only and another yielded a brucella colony by the centrifugation procedure only.

The swabbing procedure identified 18 of the 26 positive samples while the plating procedure identified 14. The centrifugation procedure identified 22 and filtration 21 of the infected samples. Of the total number of isolations for each procedure, only the plating procedure had significantly fewer isolations than the centrifugation procedure ($p<0.05$). There were no significant differences among the swabbing, centrifugation, and filtration procedures.

Table 9. Number of Brucella abortus isolations from naturally infected milk by four procedures

Number of isolations	Swabbing	Plating	Centrifugation	Filtration
11	+[a]	+	+	+
4	+	-[b]	+	+
3	-	-	-	+
2	-	-	+	+
2	+	-	+	-
1	+	+	+	-
1	-	+	+	+
1	-	+	-	-
1	-	-	+	-
49	-	-	-	-
Totals 75	18	14[c]	22	21

[a]+ = Isolation.

[b]- = No isolation.

[c]Plating total is significantly less than other totals ($p<0.05$).

The colony counts of 4 procedures for the isolation of B. abortus from naturally infected milk are shown in Table 10. The colony count data are mean values of all the plates used in a particular isolation procedure. The centrifugation and filtration procedures concentrated more organisms for subculture and identification than the other procedures. The direct plating procedure resulted in 9 contaminated attempts of the 26 isolations. Contamination did not prevent subculture from the other isolation procedures. For each milk sample, biotyping of isolates from each procedure was identical. B. abortus biovar 1 was isolated 14 times and strain 19 was isolated 12 times.

B. abortus isolation patterns in the filtration procedure are summarized in Table 11. The 5.0, 1.2, 0.8, 0.65, and 0.45um pore size filters accounted for 14, 16, 10, 12, and 7 successful isolations respectively of the total 21 isolations. The 0.45 um filter had significantly fewer isolations than the other 4 filters ($p<0.05$). The 5.0 um and the 1.2 um filters accounted for 17 of the 21 isolations (80.9%). Addition of the 0.8 um filter to the other 2 filters accounted for 19 of the 21 isolations (90.5%). Addition of the 0.65 um filter to the other 3 filters accounted for all 21 isolations. All of the isolations made from the 0.45 um filter were also made on the other filters.

Table 10. Colony counts[a] of Brucella abortus isolated from naturally infected milk by four procedures

Sample number	Swabbing	Plating	Centrifugation	Filtration and broth enrichment
14	150	TNC[b]	TNC	+[c]
28	17	Con[d]	31	+
29	1	5	1	+
33	6	20	16	+
35	-[e]	Con	1	-
37	12	12	27	30
38	20	Con	33	-
39	50	Con	31	-
42	-	-	1	+
43	1	C	1	-
44	-	-	-	+
48	15	6	TNC	+
49	-	1	1	+
50	1	6	2	+
51	23	Con	TNC	+
52	31	TNC	TNC	+
53	2	Con	5	+
54	17	-	29	+
55	-	1	-	-
56	1	1	1	+
57	-	Con	1	+
58	-	Con	-	+
64	1	4	1	TNC
69	-	-	-	+
74	2	12	47	+
75	1	2	3	+

[a]Estimated mean colony counts per plate.

[b]TNC = Too numerous to count.

[c]+ = Confluent growth (No distinguishable colonies) due to broth enrichment.

[d]Con = Contaminated.

[e]- = No isolation.

Table 11. Brucella abortus isolation patterns from broth with filter

Sample Number	Filter pore size[a] 5.0	1.2	0.8	0.65	0.45
14	-[b]	+[c]	+	+	+
28	+	+	-	-	-
29	+	-	-	-	-
33	+	+	-	-	-
37	-	+	-	-	-
42	+	+	+	+	+
44	+	+	-	-	-
48	+	+	+	+	-
49	-	-	-	+	+
50	+	+	+	+	-
51	-	-	-	+	+
52	+	+	+	+	+
53	+	+	+	+	+
54	+	+	+	+	+
56	-	-	-	+	-
57	-	-	+	-	-
58	+	+	-	-	-
64	+	+	+	+	-
69	-	+	-	-	-
74	+	+	+	+	-
75	+	+	-	-	-
Totals 21	14	16	10	12	7[d]

[a]Measured in um.

[b]- = no isolation

[c]+ = isolation

[d]Total isolation from .45 filter broth was significantly less than the 1.2 filter broth ($p<0.05$).

The colony counts of B. abortus plated from the filtrate after filtration with various pore size filters was used to measure the efficiency of this isolation procedure to retain the brucella organisms on the membrane filter. These data are summarized in Table 12. The colony counts for each filter indicate organisms that were able to pass through that particular membrane filter. No brucella organisms were detected in the filtrate of the 0.45 um filter. Only 1 colony forming unit was detected in the filtrate of the 0.65 um filter. The larger pore size filters allowed some brucella organisms to pass through. There was a reduction in the number of organisms recovered as the filter pore size was reduced. The total number of isolations from the filtrates after the 0.8, 0.65, and 0.45 um filters was significantly fewer than the isolations from the 5.0 um filter filtrate ($p<0.05$).

Table 12. Colony counts of Brucella abortus plated from filtrate after filtration with various pore size filters

Sample Number	Colony Counts of filtrate after filter 5.0	1.2	0.8	0.65	0.45
14	-[a]	-	-	-	-
28	3	1	-	-	-
29	1	-	-	-	-
33	4	1	-	-	-
37	1	1	-	-	-
42	4	-	-	-	-
44	-	-	-	-	-
48	12	9	7	-	-
49	-	-	-	-	-
50	-	1	-	-	-
51	52	19	-	-	-
52	23	9	3	-	-
53	3	3	1	1	-
54	30	16	1	-	-
56	-	-	-	-	-
57	-	-	-	-	-
58	-	-	-	-	-
64	1	-	-	-	-
69	-	-	-	-	-
74	-	-	-	-	-
75	-	-	-	-	-
Totals 21	11	9	4[b]	1[b]	0[b]

[a]- = No isolation.

[b]Total number of isolations of filtrate after .8, .65, and .45 was significantly less than 5.0 filtrate ($p<0.05$).

DISCUSSION

As a result of these studies, a procedure has been devised which permits the filtration of milk samples without apparent damage to *B. abortus* which might be present. The addition of trypsin and Tween 80 to milk samples resulted in physical changes enabling their passage through bacteria-retaining filters. A selective broth was utilized to recover the brucella organisms from the filters for identification.

The studies were designed to estimate brucella viability after treatments required for milk filtration. The viability results are estimates because of differences between experimentally-infected milk suspensions and naturally-infected milk. In naturally-infected milk, some of the brucella organisms are located in the somatic cells whereas the experimental milk suspension offers no such protection from the prefiltration treatments. The milk used for the suspensions was heated to control contaminantes and some of the milk fat removed prior to making the brucella suspensions. These changes to the milk components could have decreased the brucella viability compared to brucella viability in naturally-infected milk by decreasing the protective components of milk. However, the investigation of prefiltration treatments was limited to experimentally-infected milk due to the unavailability of sufficient quantities of naturally-infected milk for each brucella culture. These differences between experimental milk suspensions and naturally-infected milk have affected the four variables evaluated: surfactant, treatment temperature, treatment duration, and enzyme.

Of the two surfactants evaluated, Tween 80 and Triton X-100, only the Tween 80 had no effect on brucella viability. Because both concentrations of Triton X-100 caused growth inhibition of B. abortus biovar 2 on the disk diffusion test, this surfactant was not utilized in further evaluations. Preliminary work adapting the bacterial enumeration procedure to filtering larger amounts of milk, indicated no apparent advantage of filtering capabilities utilizing either surfactant. The Triton X-100 was unsuitable for filtration of B. abortus from bovine milk due to B. abortus biovar 2 growth inhibition and only equal filtering capabilities compared to Tween 80. Between the two dilutions of Tween 80 (1.0% and 0.1%), neither caused inhibition of brucella growth, but the 1.0% dilution of Tween 80 resulted in greater filtration facilitation. The optimal surfactant for the filtration of bovine milk was 1.0% Tween 80.

The maximum parameters of the heating and the duration of the exposure having no effect on brucella viability were difficult to establish due to the conflicting results. At 45°C, B. abortus strain 19 was significantly affected, yet 50°C for 10 and 20 minutes did not significantly affect viability. B. abortus biovar 1 was more resistant to heat treatment than biovars 2 and 4. Although B. abortus biovar 2 and 4 were significantly affected by the 50°C treatment for 10 minutes, reducing the treatment duration to 5 minutes would allow filtration of the milk and yet not kill all brucella organisms. The 5 minute 50°C heating is below the limits on the thermal death curves for brucella organisms[59]. In an effort to set prefiltration treatment parameters away from limits which could

potentially decrease brucella viability, the heating was established at 50°C for 5 minutes prior to filtration.

The estimations of brucella viability following 25% trypsin treatment were conflicting. While milk suspensions of B. abortus biovars 1, 2, and 4 lost viability after trypsin treatment, B. abortus strain 19 milk suspensions did not. Although treatment of the brucella milk suspensions with 25% trypsin is detrimental for some B. abortus cultures, the trypsin treatment is required for filtration of 10 ml of milk. The loss of brucella viability in experimentally-infected milk suspensions after trypsin treatment may only be an approximation of brucella viability in naturally-infected milk due to the brucella organisms located in the milk somatic cells of the naturally-infected milk. Other individual sample variables such as fat globules and the milk protein, casein, if in greater concentration than in the prefiltration studies, may protect brucella organisms. There is the possibility that the trypsin treatment could cause the rupture of the somatic cells releasing the intracellular brucella organisms and increasing the opportunity for isolation of B. abortus.

The combined effects of the treatments were measured to provide information on the susceptibility of the B. abortus biovar 1 to the trypsin treatments. Since isolations of B. abortus biovar 1 isolates account for 70 to 80 percent of all isolations[45], this is a significant concern. The combined treatments did not have a detrimental effect on B. abortus biovar 1 viability. The trypsin's detrimental effect on brucella viability was minimized by the other treatments. Although conflicting

information was gathered concerning the affect of the trypsin treatment on brucella viability, there was enough information supporting its lack of effect coupled with the differences between experimentally- and naturally-infected milk to justify proceeding with the investigation of the filtration procedure.

The comparison of B. abortus isolation procedures from naturally-infected milk provides the key information for assessing these procedures. Although the number of organisms in the sample is not known, making it impossible to measure sensitivity, there are a few considerations to be taken when evaluating the data. All of the milk samples had been frozen and thawed several times. This may have released many of the intracellular brucella organisms making the plating and swabbing procedures more likely to isolate because there was no need for concentration of the organisms. The repeated freezing and thawing would decrease the number of viable brucella organisms to the point that some isolation attempts were futile. The quality of the milk components was also effected. Some samples had separated cream and after centrifugation some sediments were thick and dry. Some milk samples were similar to fresh milk samples with regard to amount and quality of the milk components.

The isolation patterns of the four procedures demonstrated the randomness of brucella isolation and the difficulty in concentrating the brucella organisms. From the colony counts per isolation, there was an appearance that small numbers of the brucella organisms were isolated for many of the samples. This is evident in the milk sample number 55 which

yielded only 1 colony by the plating process. This isolation must be balanced against the 9 contaminated isolation attempts that were successfully isolated by other procedures. All of the swabbing isolations were duplicated by one of the other procedures.

The centrifugation procedure only had one isolation not duplicated by another procedure. The filtration procedure had 3 isolations that were not duplicated. By combining the centrifugation and filtration isolation results, 25 of the 26 isolations were made.

Contamination affected the isolation results only in the plating procedure. The high numbers of non-brucella organisms overwhelmed the selective media's antibiotic capabilities when 1 ml of the milk sample was placed on the media. The swabbing and centrifugation procedures utilize a cotton swab which may control the contamination by restricting the amount of milk sample on the media to less than 1 ml. The filtration procedure limited most of the contamination by the selective antibiotics in the broth and the solid media. The broth had an advantage in that if contaminated, a small portion of the broth was used to inoculate another 5 ml of fresh broth. This decreased the effect of contamination and prevented the brucella organisms from converting from the smooth colony type to the rough colony type which is difficult to biotype. The broth enrichment accounted for the large number of brucella organisms isolated by the filtration process.

There was one particular non-brucella organism isolated frequently by the filtration procedure that mimicked the appearance of brucella growth. In the early stages of growth, this organism had the same blue coloring

and size of brucella but in later growth stages the colonies were pink. The organism had a generation time similar to brucella. This organism was identified as _Pseudomonas mesophilia_ which has been isolated from rye grass, rumen of cattle, and soil, and it has been implicated as a laboratory media contaminant. However, medium checks by plating of the broth with and without the filters, the Tween 80 and trypsin solution, did not isolate _P. mesophilia_. The only plausible source was the milk sample. This contaminant was overcome by inoculation of fresh broth and did not affect the filtration results.

The efficiency of the filtration process was evaluated by noting which pore size filters were able to capture the brucella organisms. The larger pore size filters retained the brucella organisms. The rate of flow through the larger filters was much faster then through the smaller filters. The 0.45 um pore size filter was the most susceptible to clogging. More milk sample passed through the larger pore filters without clogging the filter. This increases the opportunity for isolation of _B. abortus_ because of the larger sample size examined.

The efficiency of the filtration process for retaining brucella organisms was measured by the number of organisms isolated after plating 1 ml of the milk sample post-filtration. The 0.45 um pore size filter passed no detectable brucella organisms. The higher colony counts of the filtrate after the 5.0 um pore size filter were from samples that were heavily infected with _B. abortus_ organisms as indicated by other isolation procedures. Sample 53 had a different pattern from the other post-filtration patterns. Although there were not many brucella organisms

isolated, there were organisms that passed through the 5.0, 1.2, 0.8, and 0.65 um filter. Based upon the majority of samples that had few isolations after the 0.65 um filter and no isolations after the 0.45 um filter, isolation of B. abortus by filtration retains all of the organisms.

The number of days to isolate brucella organisms after filtration was an indication of the complexity of this procedure compared to conventional centrifugation. The average time of 6.2 days was comparable to the 7 day incubation of inoculated plates after centrifugation. The filtration procedure had an advantage in the larger number of colonies available for subculture as compared to the centrifugation plates. If there was contamination, the filtration procedure took longer to resolve by re-inoculation of the broth, however, the centrifugation procedure had no options for contaminated plates but to report a no test. The filtration process, therefore, takes longer to culture B. abortus but has greater capabilities for isolating a large number of brucella organisms and controlling contamination.

The isolation of B. abortus from bovine milk is often difficult due to the small number of organisms available and the overgrowth of contaminants masking the brucella growth. This investigation was able to develop the filtration procedure and compare it to the centrifugation, swabbing and plating procedures as a means of overcoming these obstacles to isolating B. abortus from bovine milk. The filtration procedure, as developed, was not detrimental for brucella viability and provided an opportunity to concentrate the organisms prior to enrichment with broth. The filtration

process was a long, complicated method, susceptible to filter clogging, and no more statistically sensitive to isolating B. abortus than conventional centrifugation. Even though there were no statistical differences in the two isolation rates the filtration procedure had 3 successful brucella recoveries not duplicated in the three other procedures. The randomness in isolating B. abortus from milk would dictate that more than one isolation procedure be employed to assure a thorough examination of the milk sample. The plating procedure was too susceptible to contamination to be useful. The swabbing procedure was quick, easy, and the small volume minimizes contamination but there is no opportunity to release the intracellular brucella or concentrate the brucella organisms. The conventional centrifugation procedure had more isolations of B. abortus than the other procedures and the method is simple. These two advantages would support the recommendation of utilizing the centrifugation procedure for the initial isolation attempt of B. abortus from bovine milk. The filtration procedure could be utilized as a confirmatory method when no isolation was made on the initial attempt or the sample quality would prevent centrifugation. Examples of milk samples that are not suitable for centrifugation are non-lactating udder secretions and insufficient samples of less than 10ml. The filtration process could concentrate the brucella organisms by diluting the viscous secretion with Tween 80 as a prefiltration treatment rather than swabbing this sample. Samples with insufficient size after centrifugation lack the cream and sediment to spread upon the selective media. The isolation attempt can then utilize the advantages of the

filtration procedure's control of contamination, concentration of brucella organisms, and enrichment by the selective broth.

The prefiltration reagents are all stable in storage so the procedure can be utilized without lengthy preparation. When isolation of B. abortus from bovine milk is attempted, the combination of the centrifugation and the filtration procedures will be the most thorough examination of the milk sample possible.

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