VIBRIO FETUS CONTROL IN STORED SEMEN

By

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INTRODUCTION

The cow is a basic element of a dairy operation and it is of utmost importance that she reproduce efficiently. When a cow fails to reproduce satisfactorily, the economic status of the dairyman is affected. Various factors may arise which affect the reproductive efficiency of a cow. Included in the host of causative factors is the organism known as _Vibrio fetus_. The importance of _Vibrio fetus_ infection as a cause of infertility in cattle has been increasingly appreciated in recent years.

The infectious disease known as bovine vibriosis was reported by MacFadyean and Stockman (30) in 1913. The causative organism was studied intensively in 1917-1923 by Smith and co-workers (44,45) and given the scientific name of _Vibrio fetus_.

For many years the disease was regarded as one of the minor reproductive diseases of cattle. During the early investigational period, abortion was generally regarded as the only significant observable symptom of the disease. Since that time, investigations by Plastridge and other researchers have renewed interest in the disease and other symptoms have been revealed (1,19,28,39). Plastridge and his co-workers (38) and Gauthier and Cheve (19) observed that abortion is still a criteria for vibriosis. However, Plastridge and his associates (39) reported that vibriosis may impair breeding efficiency by lowering conception rates, as well as by causing abortion. Stegenga (46) observed that irregularity of the estrous cycle was directly associated with a lowered conception rate.

Thus, delayed conception and irregular estrous cycles are also common manifestations of the infection.

Apparently, the disease may be spread in different ways. In recent years, attention has centered on the bull as a principal potential carrier and disseminator of vibriosis.

This work was conducted to determine the effects of the antibiotics dihydrostreptomycin sulfate and procaine penicillin G on *Vibrio fetus* organisms purposely instilled into bovine semen which was to be stored by conventional methods. Particularly, information was desired as to the survival of *Vibrio fetus* in the presence of these antibiotics when held for 1 hour at 30°-35° C..

REVIEW OF LITERATURE

Numerous investigators (7,20,37,38,39,45,47) have shown that the bull can be an important factor in the dissemination of vibriosis.

Plastridge and Williams (38) were associated with herds which were known to be infected with *Vibrio fetus*. During the course of their observations, they noticed a slow and progressive spread of the disease in the herds under study. They suggested that the bull was the cause of the spreading of the disease.

Binns and Fincher (7) were successful in the isolation of *Vibrio fetus* from the preputial mucus and semen of bulls which were known to be infected. The organism was found readily on bacteriological examination of the preputial mucus and semen. The authors considered that it should not be necessary to take more than three samples of preputial mucus from an infected bull to demonstrate *Vibrio fetus*.

Willet, *et al*. (48) conducted an experiment with seven bulls known to be infected with *Vibrio fetus*. The semen from these bulls, as used in artificial insemination, produced a greater embryonic mortality and lower conception rates than semen from healthy bulls.

Hughes (20) examined bulls in two large artificial insemination centers to determine the incidence of *Vibrio fetus* infection. One group of thirty-nine bulls had never served a cow in natural service, whereas, a second group of one hundred and four bulls had, at some time, been used in natural service. The incidence of infection was practically the same

for both groups - 56.4% and 56.7% respectively. From this information, Hughes concluded that Vibrio fetus could be transmitted from bull to bull as well as from bull to cow and vice versa. He further substantiated this conclusion by observation of the management practices at the studs. He suggested that transmission occurred at the time of collection by penile contact with the rear quarters of the dummy which was used for the collection of all bulls in the stud. Hughes further stated that improper sterilization of the artificial vagina could also be a cause of the spread of Vibrio fetus from bull to bull in an artificial insemination center.

Plastridge, et al. (37) examined samples of semen and preputial washings from 149 bulls used in four artificial breeding centers for the presence of Vibrio fetus. Blood agar plates, incubated in 5% oxygen, 10% carbon dioxide and 85% nitrogen were more efficient in detecting Vibrio fetus than other cultural methods. Forty-one percent of the bulls were infected with Vibrio fetus. Of these, 39% were detected in one test, 67% in two tests and 95% by three tests of the preputial washings. Of the 544 semen samples examined, 27% yielded Vibrio fetus. The authors stressed the danger of transmission of Vibrio fetus by ordinary methods of semen collection.

Artificial insemination has also been shown to be a factor in the spread of the disease.

Stegenga (46) reported the results of a 7 year investigation concerning enzootic sterility in the Netherlands. His results indicated that enzootic sterility in the Netherlands was due to Vibrio fetus. Infection with this microorganism was found only in cattle that had been bred by bulls known to have the disease. This sterility was also spread

by artificial insemination, and in one outbreak, abortions were attributed directly to Vibrio fetus. The author stated that the disease was transmitted by infected bulls which appeared to be normal and whose semen appeared to be normal.

Rasbech (41) found in Denmark that the use of semen from infected bulls had resulted in widespread dissemination of the disease. Using the vaginal mucus agglutination test on the cows, he was able to disclose which bulls were spreading vibriosis. He recommended that suspected carrier bulls be bred to several virgin heifers as a diagnostic means of revealing Vibrio fetus carrier bulls.

Easterbrooks and Plastridge (12) conducted a study from which they concluded that Vibrio fetus could be transmitted in diluted semen and also by contact with infected animals. They used two groups of six heifers each. One group was artificially inseminated with semen in "Ortho" liquid diluter. The second group was the same except that it had viable Vibrio fetus added to the mixture. The heifers bred with infected semen required 3.8 services per conception. The heifers bred with control semen required 1.8 services per conception. However, two of these returned to heat at 58 and 118 days. Abortion was apparent and tests disclosed that both had become infected with vibriosis. All of the heifers in both groups had been in contact with each other throughout the trial. Therefore, the authors concluded that the Vibrio fetus organism could be transmitted through semen and consequently through contact.

Lawson and MacKinnon (25) reported that 22 of 24 heifers became infected when served by an infected bull or artificially inseminated with infected semen.

Hughes (22) artificially inseminated 30 heifers with Vibrio fetus infected semen which was diluted 1:4 and which contained no antibiotics. Twenty-six or 86.6% of the heifers became infected with vibriosis. When infected semen was similarly diluted 1:25 in extender, without antibiotics, and used to inseminate 29 heifers, only four or 13.8% became infected. The author stated that this illustrated the value of dilution in reducing the incidence of infection.

McEntee, et al. (28) experimentally produced vibriosis in virgin heifers. One group of heifers was inseminated either with semen from infected bulls or artificially inoculated semen. The control group was bred with uninfected semen. Those heifers bred with infected semen required a median of five (range 1-16) services per conception. The control group required a median of one (range 1-3) service per conception.

Considerable work has been done recently regarding the use of antibiotics in semen diluters. Several authors (4,5,6,15,16,23) have indicated the usefulness of antibacterial agents in diluted semen.

Foote and Bratton (15) established the fact that the addition of antibiotics to diluted semen from bulls of low levels of fertility results in a significant increase in the rates of conception. They stated that this effect was probably due largely to the inhibitory or lethal effect on the bacteria present. A question of prime importance was whether these antibiotics were able to kill Vibrio fetus and render the semen safe for use in artificial insemination without having a harmful effect on the spermatozoa.

Adler and Rasbech (1), using 11 Vibrio fetus infected bulls, compared 60-90 day non-return rates using untreated diluted semen and

diluted semen treated with 5000 mcg. of streptomycin per ml.. Of the 11 bulls used in this study, nine showed a marked improvement in fertility when the semen was treated. The non-return rate for the untreated semen was from 41.9% to 64.0%, whereas, the non-return rate for the treated semen was from 58.5% to 66.1%. A comparison of using 5000 mcg. and 1000 mcg. of streptomycin per ml. of diluted semen showed no significant difference due to level of streptomycin.

Frank and Bryner (17) found that _Vibrio fetus_ was not recovered from cows and heifers artificially inseminated with treated semen from infected bulls. One thousand units of penicillin and 1000 mcg. of streptomycin per ml. were added to the diluter. The diluter was warmed to body temperature before the addition of the semen and then cooled to 5° C.. The diluted semen was not used until at least 5 hours after preparation.

McEntee, _et al_. (29) reported that vibriosis did not develop in 94 heifers inseminated with treated diluted semen from infected bulls. The antibiotic treatment consisted of 500 units of penicillin, 500 mcg. of streptomycin, and 3 mg. of sulfanilamide per ml. of diluted semen. The semen was diluted at least 1:25, cooled to 5° C., and held 6 hours before use. The authors found no vibriosis in herds serviced with semen that had been handled in this manner.

An experiment was conducted by Morgan, _et al_. (31) in which three treatments were used: (I) undiluted semen with no antibiotics; (II) semen diluted 1:5 with egg yolk-citrate buffer and containing 500 mcg. of streptomycin per ml.; and (III) semen diluted 1:50 with egg yolk-citrate buffer containing 500 mcg. of streptomycin per ml.. After dilution, the semen samples were held at 5° C. for 2 hours before being used for insemination. One heifer was bred with semen from Treatment I, three

were bred with semen from Treatment II and two were bred with semen from Treatment III. One heifer from each group became infected with vibriosis as shown by recovery of the organism from uterine biopsies, samples of estrual mucus, and positive reactions to the mucus agglutination test. From these data, the authors stated that neither dilution rate nor the addition of 500 mcg. of streptomycin per ml. would prevent heifers from becoming infected with Vibrio fetus.

Lawson, as cited by Morgan, et al. (31) infected five of 23 heifers by insemination with treated, diluted semen. The semen was diluted 1:25 and treated with 500 units of penicillin, 500 mcg. of streptomycin and 3 mg. of sulfanilamide per ml.. The processed semen was held at room temperature for 2 hours before being stored at 4° C..

Easterbrooks, et al. (13) bred eight heifers with diluted semen which was treated with streptomycin and artificially infected with Vibrio fetus cells. None of the heifers developed vibriosis. The authors made the following recommendations: (a) use 500 to 1000 mcg. of streptomycin per ml. of warm (30°-35° C.) diluter; (b) dilute the semen 1:10 and let it set for 1 hour to cool at room temperature; and (c) place it in the refrigerator for 4 to 6 hours before use.

Lawson and MacKinnon (24) treated diluted, infected semen with 1000 units of penicillin and 1000 mcg. of streptomycin per ml.. Six heifers were bred artificially with the diluted, infected semen and four of them became infected with vibriosis. The authors concluded that neither of the antibiotics was effective in controlling vibriosis. They added the antibiotics to the semen shortly before insemination. A definite time of exposure was not stated, nor was mention made of the length of time the treated semen was held at or above room temperature before it was cooled to 5° C..

Several in vitro experiments have been performed to determine the usefulness of antibiotics in controlling the <u>Vibrio fetus</u> organism in diluted semen.

Roberts (43) and Easterbrooks, <u>et al</u>. (11), in their preliminary observations, indicated that the commonly used addition of 500 units of penicillin and 500 mcg. of streptomycin per ml. of semen diluter would inactivate <u>Vibrio fetus</u>. Mundt (32) observed that 1000 I. U.'s of penicillin inhibited growth in six out of ten <u>Vibrio fetus</u> cultures. Streptomycin, at 50 mcg. per ml., inhibited the growth of most of the strains. Jennings (23) stated that <u>Vibrio fetus</u>, when grown on blood agar plates, was not sensitive to penicillin in concentrations up to 100 units per ml..

Prier (40) reported that 0.6 to 39.0 units of penicillin per ml. would inhibit <u>Vibrio fetus</u> growth in thiol, depending on the strain used. He also found that 0.3 to 78.0 mcg. of streptomycin per ml. would inhibit the growth of <u>Vibrio fetus</u>. No definite time of exposure of the organism to the antibiotics was stated, nor were the organisms removed from the presence of the antibiotics. Orthey and Gilman (33) contended that if the organism was not completely removed from contact with the antibiotics, it would be impossible to determine whether the action was bactericidal or bacteriostatic.

Plastridge and Easterbrooks (36) conducted an in vitro experiment to test the effect of antibiotics on <u>Vibrio fetus</u> in diluted bull semen. They reported that 500 units of penicillin had no adverse effects on <u>Vibrio fetus</u>, whereas, 500 mcg. of streptomycin brought about death in 24 hours at 37° C.. The addition of 500 mcg. of streptomycin per ml. did not destroy <u>Vibrio fetus</u> at 5° C.. However, the authors stated that

if the organism was present in semen treated in this manner, it would not be capable of causing infection because body temperature and the stability of streptomycin would prevent infection. The authors stated that the addition of 1000 mcg. of streptomycin per ml. of diluent would render Vibrio fetus infected semen noninfective, provided the diluted semen was allowed to stand at least 1 hour at 37° C..

Albertsen (3) tested the bactericidal effects of streptomycin, penicillin, neomycin, aureomycin, terramycin, and chloromycetin against Vibrio fetus and other infectious organisms. At 25° C. and 37° C., streptomycin appeared to be the superior antibiotic for eliminating pathogens from semen. Both streptomycin and terramycin exhibited bactericidal effects at these temperatures, while the other antibiotics exhibited static effects. Albertsen stated that streptomycin had a poor killing effect on Vibrio fetus at 5° C.. He also stated that semen cooled according to the procedure of Foote and Bratton (14) could easily contain viable organisms.

In another study, Albertsen (2) concluded that the effect of antibiotics was greatly dependent on temperature, growth phase of the bacterium at the moment concerned and on environmental conditions. Using 1000 mcg. of streptomycin per ml. of semen diluter on four strains of Vibrio fetus, he found that the bactericidal effect of streptomycin rose with increasing temperature. At the temperature of 5° C., Vibrio fetus had a half life of 293 minutes, at 25° C. a half life of 75 minutes, and at 30° C. a half life of only 6 minutes. He suggested that, at 5° C., a semen diluter containing 1000 mcg. of streptomycin per ml. would not have a sufficient bactericidal effect on Vibrio fetus. Too, the author indicated that streptomycin, at the level used, could be expected to have a

sufficient bactericidal effect on Vibrio fetus only at temperatures ranging from 25° to 30° C..

Orthey and Gilman (34) found that both penicillin and streptomycin had bactericidal action against Vibrio fetus in artificially infected semen. However, streptomycin exhibited greater bactericidal action than did penicillin. They observed that 500 units of penicillin and 500 mcg. of streptomycin per ml. of diluted semen killed all Vibrio fetus organisms in concentrations up to 225 X 10^7 organisms per ml., when exposed for 6 hours under the temperature conditions recommended by Foote and Bratton (14). This consisted of diluting the semen 1:4, placing it in a tumbler of water at 30° C., and placing in a refrigerator at 5° C.. The semen was removed from the water after 20 minutes and allowed to stand in cold room air (5° C.) for 55 minutes before dilution to final volume. Approximately 6 hours were required to reach a temperature of 5° C.. Since the number of organisms used in this study was many more than found in naturally infected semen, this combination of antibiotics seemed capable of rendering innocuous all Vibrio fetus organisms that might be present in naturally infected semen. To test the validity of this line of thinking, Hughes (20) cultured 201 naturally infected semen samples at the end of 1 and 6 hours of exposure to 500 units of penicillin, 500 mcg. of streptomycin, and 3 mg. of sulfanilamide per ml. in the diluter. The organism was not recovered from any of the samples after 1 or 6 hours of exposure to the antibiotics.

Orthey and Gilman (33), in a later study, tested the action of penicillin, streptomycin, and sulfanilamide against heavy suspensions of Vibrio fetus. They reported that streptomycin was bactericidal, but penicillin and sulfanilamide were not bactericidal. The streptomycin

was most effective when the diluter-antibiotic-bacterial mixture was incubated for 1 hour and then refrigerated for the remainder of the observation period. The minimum bactericidal concentration of streptomycin was 2000 mcg. per ml.. The addition of penicillin and sulfanilamide did not increase the efficiency of streptomycin.

DiLiello, et al. (9) studied the effects of several antibiotics singly and in combination against Vibrio fetus and bovine sperm. Of the antibiotics tested, dihydrostreptomycin and/or streptomycin at the 500 mcg. level gave best control of Vibrio fetus and was the least harmful to sperm. Penicillin, in a concentration of 500 units per ml., was combined with 250 mcg. of streptomycin, 500 mcg. of dihydrostreptomycin, and 500 mcg. of neomycin. This combination did appear to exert a more pronounced effect against Vibrio fetus than any antibiotic acting alone at similar levels.

Streptomycin has emerged as the most important antibiotic to use in treating diluted semen to control the spread of vibriosis. However, recent studies have revealed that the use of glycerol in diluters enables Vibrio fetus to escape the controlling effect of streptomycin.

The survival of Vibrio fetus in frozen semen containing 1000 units of penicillin and 500 mcg. of streptomycin per ml. was reported by MacPherson and Fish (27). Tubes of diluted semen were placed in a water bath at 30° C.. The water bath was then placed in a refrigerator for cooling to 5° C.. Portions of the diluted treated and untreated semen were glycerinated, frozen, and stored at -79° C.. Cultural tests made after 3 and 7 days revealed that Vibrio fetus survived at 5° C. and at -79° C. in both the treated and untreated semen. However, the authors were able to recover viable organisms only after residual penicillin and streptomycin were removed by washing.

Preliminary trials by Dunn, et al. (10) had indicated that glycerol was interfering with the action of streptomycin. They processed semen for freezing in yolk-citrate diluter containing 7% glycerol, 500 units of penicillin and 500 mcg. of streptomycin per ml.. The authors were able to isolate positive Vibrio fetus cultures from ampules of thawed semen. To secure further information on this problem, the authors conducted a study to test the effectiveness of various holding periods in the presence of different levels of penicillin, streptomycin, and terramycin. Naturally infected semen from two large bull studs was processed and frozen by routine methods. Positive cultures of Vibrio fetus were found, even after contact with 2000 mcg. of streptomycin per ml. for 4 hours prior to the addition of glycerol. In another experiment, using artificially infected semen, they observed that Vibrio fetus organisms, rendered culturally nonviable by contact with streptomycin, became culturally viable and able to reproduce after the addition of glycerol. This condition existed even if a 48 hour holding period at 5° C. were permitted before the addition of glycerol.

Hughes (21) stated that the release of the organisms from the action of streptomycin was not related to freezing, but was related to some chemical-physiological reaction that took place in the culture media.

This problem concerning the release of Vibrio fetus organisms from the antibiotics' action was serious due to the fact that Leidl and Mahrlu (26) and Hughes (21) reported that Vibrio fetus was able to withstand deep freezing to -79° C.. Therefore, it was clearly possible that vibriosis could be spread through the use of frozen semen.

It must be pointed out that MacPherson and Fish (27) did not hold the treated semen for one hour at 25° C. to 35° C. as had been previously

recommended by Easterbrooks, et al. (13). Dunn, et al. (10) and Hughes (21) did not stipulate the temperature conditions under which they froze the semen in their studies. Plastridge (35) advanced conditions that should be met before it could properly be concluded that antibiotic treatment was ineffective in controlling vibriosis in semen processed by freezing. He asserted that the following test should be conducted when using the semen of known infected bulls. The diluted, treated semen should be held for 1 hour at 25° C. to 35° C.. The semen should be tested for infectivity by inseminating virgin heifers and then testing the heifers by cultural and cervico-vaginal mucus agglutination tests.

Recently, VanDemark and Sharma (47) demonstrated the successful maintenance of fertility of bull semen for 6 days at room temperature. This was accomplished by incorporating carbon dioxide into a suitable diluent and hermetically sealing the diluted semen in ampules.

With the introduction of this method of semen storage, the question naturally arose concerning the possibility of transmitting infectious organisms through the use of such semen. Rhoades and VanDemark (42) studied the survival of Vibrio fetus and other infectious organisms in the Illini Variable Temperature diluter (hereafter referred to as the IVT diluter) at 29° C.. The diluter was prepared as reported by VanDemark and Sharma (47) and 25 million sperm per ml. were added. The authors used two levels of antibiotics: (a) penicillin at 500 and 1000 I. U.'s per ml.; and (b) streptomycin (dihydrostreptomycin sulfate) at 500 and 1000 mcg. per ml.. Both levels of the antibiotics, and a control of diluent without antibiotics, were inoculated with 100,000 Vibrio fetus organisms per ml.. These samples were checked at 0, 2 and 4 days after incubation at 29° C.. To check the samples for growth of Vibrio

fetus, they were first poured into a tube containing 5 ml. of sterile saline solution and centrifuged at 3000 rpm for 30 minutes. A loopful of sediment was transferred to a tryptose crystal agar plate. The remaining portion of the sediment was added to 6 ml. of thiol broth (Difco). The agar plates and tubes of thiol broth were then incubated at 37° C. in an atmosphere of 10% carbon dioxide. Vibrio fetus was not viable at either level of antibiotics at 0, 48, and 96 hours after incubation at 29° C.. The organism was viable in all of the control samples. The authors concluded that proper antibiotic treatment of bovine semen stored at room temperature in the IVT diluter would effectively prevent the spread of vibriosis through the use of such semen.

EXPERIMENTAL PROCEDURE

The semen for this series of studies was secured from an Ayrshire bull, No. 23, one of a group of experimental bulls in the Oklahoma State University dairy research facilities. A dilution rate of 1:25 was used throughout these studies.

The types of semen storage to be studied were divided into four categories and designated as Treatments I, II, III, and IV. Each of the treatments employed the use of a solution of sodium citrate-egg yolk as the vehicle for the semen and antibiotics to which viable _Vibrio fetus_ organisms were to be added.

Treatment I involved the storage of diluted semen by freezing to -79° C.. The diluter for this treatment consisted of 80% of a 2.9% sodium citrate solution, 20% egg yolk and 7.5% glycerine. To each ml. of diluted semen was added 500 units of procaine penicillin G and 500 mcg. of dihydrostreptomycin sulfate. After collecting the semen, a dilution of approximately one-half of the final volume (at least 1:12) was made. The semen was then allowed to stand at room temperature for 1 hour in a beaker of warm water (30°-35° C.). The beaker containing the diluted semen was then placed in the refrigerator for 5 hours. The other one-half of the diluter was prepared to be 15% glycerine by volume so that the final concentration would be 7½% in the glycerolated, diluted semen. At the end of the cooling time (6 hours after the original one-half dilution), the cooled glycerolated diluter was added in four steps, 10 minutes apart in 10%,

20%, 30%, and 40% portions respectively. Cooled vials were then filled and placed in the refrigerator and allowed to equilibrate for 18 hours. Freezing was then accomplished by immersing the vials in a 99% isopropyl alcohol solution at 5° C.. Small pieces of dry ice were added to lower the temperature 1°-2° C. per minute. At -15° C., the rate was increased to 3°-4° C. per minute and at -70° C., the vials were removed and stored at -79° C. in a dry ice chest in a deep freeze. To thaw, the vials were removed from the deep freeze and immersed in ice water at 5° C. for 5-10 minutes.

Treatment II was the storage of diluted semen by refrigeration at 5° C.. The diluter consisted of 66 2/3% of a 2.9% sodium citrate solution and 33 1/3% egg yolk, with 1000 units of procaine penicillin G and 1000 mcg. of dihydrostreptomycin sulfate per ml.. The samples from this treatment were diluted at the rate of 1:25 and allowed to incubate in a beaker of warm water (30°-35° C.) for a 1 hour period. After this 1 hour cooling period, the beaker was then placed in the refrigerator for cooling to 5° C..

Treatment III was the same as Treatment II with the exception that the samples were placed immediately in the refrigerator after dilution. The purpose of this treatment was to test the validity of the recommendation that diluted semen must be given a 1 hour cooling period at or above room temperature to destroy _Vibrio fetus_ organisms (13,35).

Treatment IV was the room temperature type of semen storage. The IVT diluter was prepared in the manner reported by VanDemark and Sharma (47). The diluent was composed of 20.0 gm. sodium citrate dihydrate, 2.1 gm. sodium bicarbonate, 0.4 gm. potassium chloride, 3.0 gm. glucose, and 3.0 gm. sulfanilamide, dissolved (by heating to near boiling) in a

liter of water distilled over glass. This solution was then cooled to room temperature and then saturated with carbon dioxide by bubbling the gas through it for 10 minutes (or until the pH was reduced to about 6.35). After saturation with carbon dioxide, 1000 I. U.'s of penicillin and 1000 mcg. dihydrostreptomycin sulfate per ml. of diluent were added along with enough fresh egg yolk to make a final diluent consisting of 10% egg yolk. Semen was added at a ratio of 1:25 and then sealed hermetically in 1.5 ml. glass ampules. Storage temperature for this treatment was 20°-28° C..

Streptomycin was selected as the antibiotic to be used in the attempt to control Vibrio fetus. Its usefulness in this capacity had been indicated in the literature review (13,17,20,22,36). Penicillin was used to secure inhibition of other undesirable organisms usually present in bull semen.

The materials and equipment used in these studies were prepared in accordance with accepted bacteriological procedures. Thirty-four matched test tubes were stoppered with cotton and sterilized by autoclaving. Four pairs of tubes were prepared for each of Treatments I, II, and III and five pairs of tubes were prepared for Treatment IV. Diluter-antibiotic mixtures were prepared for each particular treatment. Five ml. of the diluter-antibiotic mixture was added to one of each pair of test tubes for each treatment respectively. The tubes were prepared at night and stored overnight at 5° C.. The semen was collected the next morning and 0.2 ml. was added to each test tube of prepared diluter. At the same time, all of the tubes were inoculated with a standardized quantity (as described below) of Vibrio fetus organisms and handled thereafter as will be indicated in a later description of

the various experiments. For each tube of semen-antibiotic-*Vibrio fetus* mixture, a control tube was set up simultaneously and handled identically. None of the controls were treated with antibiotics. The suspensions of *Vibrio fetus* organisms were added to each control tube at the same time and in the same manner as the treated tubes. Treated and control tubes were then stored and treated identically. The 34 tubes described were cultured at the end of six different exposure periods. This experiment was replicated 10 times for Treatments I, II, and IV and 9 times for Treatment III.

Three strains of *Vibrio fetus*, Rilma, Lindy, and Vegor, were used in this study. Strain Rilma was obtained from W. N. Plastridge and stored on a broth culture. Strains Lindy and Vegor were isolated from two herd bulls in the Oklahoma State University dairy stud and were lyophilized immediately after isolation. No attempt was made to detect any cultural differences between these strains. The strain Rilma was so named when received from W. N. Plastridge. Strains Lindy and Vegor were named after the bulls from which they were isolated. These strains were known to be sensitive to streptomycin. The organisms were inoculated onto agar slants and incubated at 37° C. for 2-4 days in brewer jars containing 10% carbon dioxide, 85% nitrogen and 5% oxygen. Following this incubation period, the organism was harvested by adding approximately 1 ml. of sterile physiological saline to the agar slant and gently loosening the growth with a sterile wire loop. Using a Cenco Photelometer, the bacterial suspension was then standardized to a density of #5 on the McFarland nephelometer scale. This suspension was added to the prepared tubes of diluted semen in the amount of 0.2 ml. per tube.

The diluter-Vibrio fetus mixtures were cultured for viable cells after designated periods of exposure to the antibiotics. The samples from Treatment I were checked at: (a) 1 hour (just after the 1 hour cooling period); (b) 6 hours (just prior to glycerolation); (c) 24 hours (just before freezing); and (d) 30 days after freezing. Treatments II and III were each checked at 1, 6, 24, and 72 hours. The tubes from Treatment IV were checked at 1, 6, 24, 72, and 144 hours.

When the antibiotics and Vibrio fetus organisms had been in contact for the desired period of time, the following techniques were used to remove the bacteria from the action of the antibiotics and to test their viability. The tubes containing the mixtures of semen-bacteria-diluter, with or without antibiotics, were mixed well and the cotton plug secured by folding a part of it over the edge of the tube and fastening it with a rubber band. The tubes were then centrifuged for 5 minutes at approximately 750 rpm to remove the majority of the sperm. The supernatant was poured into another sterile, stoppered tube and centrifuged for 15 minutes at 3150 rpm. The supernatant was then discarded and a 5 ml. solution of nutrient broth was added and the material was resuspended and recentrifuged for 15 minutes. The supernatant fluid was again removed and another 5 ml. portion of nutrient broth was added. The sediment was resuspended by shaking and then recentrifuged for a final 15 minutes. Again the supernatant was discarded and 0.5 ml. of thiol medium (Difco) was added. The sediment was resuspended and a 4 mm. loopful of this material was inoculated onto 5% blood agar plates. Incubation followed at 37° C. in brewer jars containing 10% carbon dioxide, 85% nitrogen and 5% oxygen. The plates were inspected microscopically after 4-5 days of incubation. Those plates showing typical

Vibrio fetus colony growth were subjected to further studies. First, a simple stain, using 1% basic fuchsin, was made to ascertain that the suspected colony was actually *Vibrio fetus*. If so confirmed, then the colony was picked and placed in a test tube containing 5 ml. of thiol medium. These tubes were then incubated at 37° C. in an atmosphere of 10% carbon dioxide and were observed daily for a period of 7 days. Those showing typical growth of *Vibrio fetus* (a band of growth in the top 6 mm. of thiol) were stained with 1% basic fuchsin. Then, if the organism exhibited the typical shape of *Vibrio fetus*, it was subjected to the catalase test (8) to determine if the strain present was pathogenic. If 5 mm. of gas was produced in 30 minutes, this was considered to be conclusive evidence that the *Vibrio fetus* organism present was pathogenic.

The antibiotics used in the course of this experiment were procured from the Oklahoma State Veterinary College and stored in a refrigerator at approximately 5° C. until they were used.

RESULTS AND DISCUSSION

A summary of the effects of each treatment employed in this study is presented in Appendix Table I. Treatment III was the only treatment from which viable *Vibrio fetus* was recovered from an antibiotic treated sample.

A total of 10 replications of Treatment I was conducted during the course of this experiment. Viable *Vibrio fetus* was recovered from 39 of the 40 control samples of this treatment. After 1 hour incubation at 30°-35° C., the antibiotic treated tubes contained no viable organisms. The same pattern held true for the entire period of study for all of the 40 antibiotic treated samples of Treatment I.

These data disagree with the findings of Dunn, *et al*. (10) and Hughes (21) that glycerol somehow liberates *Vibrio fetus* from the action of streptomycin. It must be pointed out that the initial incubation time they employed was not specfically mentioned. It was inferred that the cooling procedure used was as recommended by Foote and Bratton (14). It will be recalled that all of the samples in Treatment I were subjected to a 1 hour incubation period at 30°-35° C. before cooling to 5° C. as recommended by Plastridge (35). Since viable *Vibrio fetus* was not recovered from any antibiotic sample so treated, it was logical to assume that the treatment used was more effective in the control of vibriosis than was the cooling method which has been, and perhaps still is, rather widely used.

A summary of the effects of Treatment II is presented in Table 1. As in Treatment I, viable Vibrio fetus was recovered from 39 of the 40 control samples of this treatment. Viable organisms were not recovered at any time from any of the antibiotic treated tubes. This work supports the contention of Easterbrooks, et al. (13) and Plastridge (35,36) that greater control of Vibrio fetus is secured when diluted, antibiotic treated semen is allowed to incubate for 1 hour at 30°-35° C. before refrigeration.

Treatment III was the same as Treatment II except that Treatment III did not receive the 1 hour incubation period at 30°-35° C.. The effect of this treatment is immediately apparent upon examination of Table 1. Viable Vibrio fetus was recovered from 4 of 36 antibiotic treated samples. Viable organisms were recovered from 33 of 36 control tubes. The importance of these data becomes even more apparent when compared with the results of Treatment II. No viable organisms were recovered from antibiotic treated samples in Treatment II. Since viable organisms were recovered from treated samples in Treatment III, one could conclude that the antibiotics were most effective when the diluter-antibiotic-bacterial mixtures were incubated for 1 hour and then refrigerated for the remainder of the observation period. This assumption is in direct agreement with Albertsen (2,3) whose studies clearly illustrated that the effect of antibiotics is greatly dependent upon the prevailing temperature conditions. Other workers (24,31,34) have demonstrated that incomplete control of Vibrio fetus is encountered when diluted, treated semen is not given a 1 hour incubation period at 30°-35° C.. The results from Treatment III make this problem apparent and thus it becomes imperative to incubate diluted, treated semen for 1 hour at 30°-35° C. before refrigeration.

TABLE 1

Summary of recovery of _Vibrio fetus_ by treatment and trial

	Treatment I		Treatment II		Treatment III		Treatment IV	
Trial No.	Control	Treated	Control	Treated	Control	Treated	Control	Treated
	Number of times _Vibrio fetus_ was recovered from each treatment in each trial:							
1	4	0	4	0	Not Sampled		5	0
2	4	0	4	0	4	1	5	0
3*	3	0	3	0	1	0	4	0
4	4	0	4	0	4	1	5	0
5	4	0	4	0	4	0	5	0
6	4	0	4	0	4	0	5	0
7	4	0	4	0	4	0	5	0
8	4	0	4	0	4	1	5	0
9	4	0	4	0	4	0	5	0
10	4	0	4	0	4	1	5	0
TOTALS	39	0	39	0	33	4	49	0

*Contamination was encountered in Trial 3, causing incomplete recovery of _Vibrio fetus_ from the control samples.

Treatment IV was quite effective in controlling *Vibrio fetus* as can be surmised from Tables 1, 4, and 5. *Vibrio fetus* was viable in 49 of the 50 control samples for this treatment. Viable organisms could not be recovered from any of the 50 antibiotic treated samples during the period of observation. As has been established from a comparison of Treatments II and III, it is evident that the initial temperature at which diluted, treated semen is held will materially influence the survival of *Vibrio fetus*. The initial temperature, as well as the holding temperature, for this treatment was the prevailing room temperature which ranged from 20°-28° C.. Since recovery of viable organisms from treated samples could not be effected, it appears that the use of semen stored in this method should effectively prevent the danger of spreading vibriosis. This is in accordance with the work of Rhoades and VanDemark (42) who illustrated the same fact in their studies.

A summary of the distribution of recovery by trial and time of exposure is given in Table 2. As stated previously, viable *Vibrio fetus* was recovered a total of four times from treated samples during this series of experiments. Recovery was effected once at the 6 hour, twice at the 24 hour, and once at the 72 hour interval in the observation period. As is indicated in Table 3, the Rilma strain was recovered at the 6 hour and 72 hour period. Both the Lindy and Vegor strains were recovered once each at the 24 hour sampling period. This variation in time of recovery can be explained to some extent by the fact that when the cultures of *Vibrio fetus* were secured, they varied in age from 2 to 4 days. This variation in age would undoubtedly catch the organisms in various stages of development. Therefore, some cultures would be in a more advanced stage of growth and could feasibly be recovered early in

TABLE 2

Summary of recovery of *Vibrio fetus* by trial and length of exposure interval to the antibiotics

Trial No.	Exposure Time: 1 hr.		6 hr.		24 hr.		72 hr.		144 hr.		30 days	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
	Number of times *Vibrio fetus* was recovered from each exposure time in each trial:											
1	3	0	3	0	3	0	2	0	1	0	1	0
2	4	0	4	0	4	1	3	0	1	0	1	0
3*	2	0	1	0	4	0	2	0	1	0	1	0
4	4	0	4	0	4	0	3	1	1	0	1	0
5	4	0	4	0	4	0	3	0	1	0	1	0
6	4	0	4	1	4	0	3	0	1	0	1	0
7	4	0	4	0	4	0	3	0	1	0	1	0
8	4	0	4	0	4	0	3	0	1	0	1	0
9	4	0	4	0	4	0	3	0	1	0	1	0
10	4	0	4	0	4	1	3	0	1	0	1	0
TOTALS	37	0	36	1	39	2	28	1	10	0	10	0

*Contamination was encountered in Trial 3, causing incomplete recovery of *Vibrio fetus* from the control samples.

TABLE 3

Effect of length of exposure time on strains of *Vibrio fetus*

	Exposure Time											
	1 hr.		6 hr.		24 hr.		72 hr.		144 hr.		30 days	
Strain	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of times each strain was recovered from each exposure interval:												
Rilma	15	0	15	1	15	0	11	1	4	0	4	0
Lindy	12	0	12	0	12	1	9	0	3	0	3	0
Vegor	10	0	9	0	12	1	8	0	3	0	3	0
TOTALS	37	0	36	1	39	2	28	1	10	0	10	0

TABLE 4

Effects of length of exposure time on Vibrio fetus by treatments

Hours of Exposure	Treatment I		Treatment II		Treatment III		Treatment IV	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of times Vibrio fetus was recovered from the exposure intervals in each treatment:								
1	9	0	10	0	8	0	10	0
6	10	0	9	0	8	1	9	0
24	10	0	10	0	9	2	10	0
72	-*	-	10	0	8	1	10	0
144	-	-	-	-	-	-	10	0
30 days	10	0	-	-	-	-	-	-
TOTALS	39	0	39	0	33	4	49	0

*Indicates that no observations were made at that exposure interval.

TABLE 5

Effect of treatment on *Vibrio fetus* strains

Strain Used	Treatment I		Treatment II		Treatment III		Treatment IV	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of times each strain was recovered from each treatment:								
Rilma	16	0	16	0	12	2	20	0
Lindy	12	0	12	0	12	1	15	0
Vegor	11	0	11	0	9	1	14	0
TOTALS	39	0	39	0	33	4	49	0

the sampling period. Less mature cultures would require more time for growth and maturation and would be recovered at a later period of sampling. Too, it seems logical that the phase of growth of the organism would have a bearing on the effectiveness of the antibiotics. Orthey and Gilman (33) state that streptomycin is more effective against Vibrio fetus when incubated for 1 hour at 30°-35° C. because the organism is probably in its lag phase. During this lag phase, or period of accelerated growth, the size of the cells is large, near the maximum for the species. This, according to Frobisher (18), probably represents the imbibition of water with subsequent swelling and the beginning of metabolic activity. He further states that it is likely, though not proven, that the cell walls of old or dormant cells are thicker and less elastic and permeable than those of young, actively multiplying cells. Mature and dormant cells are known to be much more resistant to most deleterious influences, including antibiotics, than young reproducing cells which are quite vulnerable to such agents. Thus, it would appear that the more mature organisms would be more able to withstand the effects of the antibiotics than would less mature organisms.

The effect of treatments on strains of Vibrio fetus used is shown in Table 5. Treatments I, II, and IV were completely effective in controlling the strains in treated samples. However, recovery was accomplished four times from a total of 36 treated samples from Treatment III. This means that viable Vibrio fetus was present in antibiotic treated samples at least 11.1% of the time. This again illustrates the usefulness of incubating treated semen for 1 hour in controlling vibriosis. This table indicates that there was essentially no difference between strains in relation to the number of times that each was

recovered. The strain Rilma was recovered twice and the other strains once respectively. It should be pointed out, as is indicated in Table 7, that strain Rilma was used a total of four times, whereas, strains Lindy and Vegor were used three times each in this study. None of the strains were recovered more than one time in any particular trial as Table 2 will indicate.

Table 4 again points out that Treatment III was the only treatment from which viable organisms were recovered from antibiotic treated samples. Too, this table shows the variability in the time of recovery that was encountered from one trial to the next. This variation in recovery from 6 hours to 72 hours is of utmost importance. It is quite evident that diluted semen stored in the refrigerator, without any incubation time, could quite feasibly contain viable Vibrio fetus organisms for the entire period that chilled semen is normally used. This fact makes it imperative that the diluted, antibiotic treated semen be incubated for 1 hour at 30° C. to 35° C. to secure maximum control of Vibrio fetus.

Appendix Table II discloses that an attempt was made to set up a definite pattern in the using of the different strains of Vibrio fetus. This pattern was closely adhered to, with one exception. In Trial 5, the Lindy strain of inoculum failed to incubate and grow properly and the Rilma strain had to be substituted in its place.

A summary of the distribution of recovery of Vibrio fetus from treated samples by trial and strain is presented in Table 6. Strain Rilma was recovered in Trials 4 and 8, while strains Lindy and Vegor were recovered once each in Trials 2 and 10, respectively. No definite pattern of recovery, either by trial or strain is present. This would

TABLE 6

Summary of recovery of *Vibrio fetus* strains by trial

Trial No.	Strain		
	Rilma	Lindy	Vegor
1	0	-	-
2	-	1	-
3	-	-	0
4	1	-	-
5	0	-	-
6	-	0	-
7	-	-	0
8	1	-	-
9	-	0	-
10	-	-	1
TOTALS	2	1	1

0 - Indicates that the strain was used but not recovered.

- Indicates that the strain was not used.

1 Indicates that the strain was used and recovered.

TABLE 7

Summary of *Vibrio fetus* strains used by trial

Trial No.	Strain Rilma	 Lindy	 Vegor
1	1	-	-
2	-	1	-
3	-	-	1
4	1	-	-
5	1	-	-
6	-	1	-
7	-	-	1
8	1	-	-
9	-	1	-
10	-	-	1
TOTALS	4	3	3

1 Indicates that the strain was used in that trial.

- Indicates that the strain was not used in that trial.

illustrate, to a certain degree, the difficulty often encountered in working with this organism. The same techniques applied in the same manner often yield quite different results. Why recovery was not possible from all of the treated samples of Treatment III can partially be explained by the fact that the organism may have been in a growth phase that was particularly resistant to the action of the antibiotics. Contamination can also be a critical factor governing the successful recovery of the organism. Contamination was encountered in Trial 3 of this study. A comparison of Appendix Table III and Tables 1, 2, 3, 4, and 5 will show the effects of this contamination. In Treatments I and III, recovery from the 1 hour control samples was not possible. This was also true of the control samples of the 6 and 72 hour replications of Treatment III and the 6 hour samples of Treatment IV. Due to the contamination of the agar plates, these samplings were almost completely overgrown. Vibrio fetus colonies were either nonexistent or were so overgrown that positive identification was not possible. Other factors such as pH, concentration of food, and atmospheric conditions have their effects also. Other reasons have been stated previously, but suffice it here to say that researchers who endeavor to reveal the secrets of this organism find it most difficult to work with.

A summary of the recovery of Vibrio fetus from the controls used in this study is presented in Tables 1, 2, 3, 4, and 8. Viable Vibrio fetus was recovered from 160 of 166 (or 96.3%) of the control samples in this study. At first thought, this may seem improbable, especially since the allusion has already been made concerning the inconsistency often encountered when working with this particular organism. However, it should be considered that the conditions for growth, in the control

TABLE 8

Summary of recovery of *Vibrio fetus* from control samples by trial and treatment

Trial No.	Treatment I	Treatment II	Treatment III	Treatment IV
	Number of times *Vibrio fetus* was recovered from the control of each treatment:			
1	4	4	Not Sampled	5
2	4	4	4	5
3*	3	3	1	4
4	4	4	4	5
5	4	4	4	5
6	4	4	4	5
7	4	4	4	5
8	4	4	4	5
9	4	4	4	5
10	4	4	4	5
TOTALS	39	39	33	49

*Contamination was encountered in Trial 3, causing incomplete recovery of *Vibrio fetus* from the control samples.

tubes, approached ideal. No antibiotics or other inhibiting agents were present, temperature for growth was ideal, the carrier solutions were of a buffered nature, and atmospheric conditions were optimum for growth. Barring outside contamination, it seems feasible that one could expect growth under these conditions. The author realized at the onset of this study that recovery of Vibrio fetus from all samples, both control and treated, that contained viable organisms was essential. With this problem in mind, extreme diligence was exerted to recover the organism from all of the samples. If there was any suggestion that the colonies being inspected were Vibrio fetus, numerous stains, with 1% basic fuchsin, and inoculations into Thiol tubes were made. At no time was positive identification of Vibrio fetus made when the tests performed were not conclusive. A high percentage of recovery of this particular organism from control samples is not a complete rarity. Rhoades and VanDemark (42) reported that viable Vibrio fetus was present in all of the control samples in their studies.

On the basis of the data obtained, it is felt that Treatments I, II, and III illustrate clearly the effectiveness of holding diluted, antibiotic treated semen for 1 hour at 30°-35° C. before cooling to 5° C.. The author also feels that it can properly be concluded from the results of Treatment IV that the antibiotics in diluted bull semen, stored at room temperature, should effectively prevent the danger of spreading vibriosis.

SUMMARY

This study was conducted to evaluate the effectiveness of various types of semen storage, with their corresponding antibiotic levels, in controlling Vibrio fetus purposely instilled into diluted bovine semen.

Four treatments that were related to methods of semen storage were used. Treatment I involved the storage of diluted, antibiotic treated semen by freezing to -79° C.. These samples were diluted, allowed to stand at room temperature for 1 hour in a beaker of warm water (30°-35° C.) and then placed in a refrigerator for cooling to 5° C.. The diluted semen was glycerolated at 6 hours, placed in 1.5 ml. vials and allowed to equilibrate for 18 hours. Freezing was then accomplished by placing the vials in an alcohol bath (5° C.) and lowering the temperature by the addition of dry ice. The vials were stored at -79° C. and observed at 1, 6, and 24 hours and 30 days after the initiation of processing.

Treatments II and III were the storage of diluted, antibiotic treated semen by refrigeration at 5° C.. These treatments were identical with the exception that all of the samples from Treatment III were placed immediately in the refrigerator after dilution. The samples from Treatment II were allowed to stand for 1 hour in a beaker of warm water (30°-35° C.) before being placed in the refrigerator. This procedure was designed to test the validity of the recommendation that diluted semen must be given a 1 hour holding period at or above room

temperature to destroy Vibrio fetus organisms (13,35). These samples were checked at 1, 6, 24, and 72 hours after the initiation of processing.

Treatment IV tested the storage of antibiotic treated semen at room temperature. This was accomplished by incorporating carbon dioxide into a suitable diluent, adding the egg yolk and semen, and hermetically sealing the solution in 1.5 ml. ampules. These samples were processed at room temperature, stored at the prevailing room temperature, and checked at 1, 6, 24, 72, and 144 hours after the initiation of processing.

For each tube of semen-antibiotic-bacterial mixture, a control tube was set up simultaneously and handled identically. The only difference between the treated and control samples was that the control samples were not treated with antibiotics.

The antibiotics employed in all of the treatment diluters were dihydrostreptomycin sulfate and procaine penicillin G.

Viable Vibrio fetus could not be demonstrated in the antibiotic treated samples of Treatments I and II that were incubated for a 1 hour period at 30°-35° C.. Viable organisms were recovered four times from a total of 36 antibiotic treated samples of Treatment III, which did not receive the initial 1 hour holding period before refrigeration. Treatment IV, which was held at room temperature from the initiation of processing, did not yield viable organisms from any antibiotic treated sample.

Viable Vibrio fetus was recovered from 160 (96.3%) of the 166 control samples in this study. Failure to accomplish 100% recovery can be explained in great part by the contamination of the agar plates in Trial 3.

A comparison of Treatments II and III is evidence that chilled semen must be incubated for the 1 hour interval to secure maximum control of *Vibrio fetus*. The results from Treatment IV indicate that there is no danger of spreading vibriosis by processing semen in the IVT diluent. This series of studies also indicates that *Vibrio fetus* infected semen can be frozen and used without danger of infection provided the diluted, antibiotic treated semen initially receives a 1 hour incubation period at 30°-35° C.. However, the author firmly believes that further work on this particular phase of semen storage should be conducted. Particularly, more comprehensive work needs to be done that would reveal the full relationship between glycerol and *Vibrio fetus*. The author feels that trials, using virgin heifers and breeding them with semen treated as stipulated, would more fully clarify this problem.

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TABLE I

Summary of recovery of *Vibrio fetus* by trial, treatment and time of exposure

	Exposure Time							
	1 hour		6 hours		24 hours		30 days	
Trial	Control	Treated	Control	Treated	Control	Treated	Control	Treated
TREATMENT I								
1	1	0	1	0	1	0	1	0
2	1	0	1	0	1	0	1	0
3*	0	0	1	0	1	0	1	0
4	1	0	1	0	1	0	1	0
5	1	0	1	0	1	0	1	0
6	1	0	1	0	1	0	1	0
7	1	0	1	0	1	0	1	0
8	1	0	1	0	1	0	1	0
9	1	0	1	0	1	0	1	0
10	1	0	1	0	1	0	1	0
TOTALS	9	0	10	0	10	0	10	0

Table I (Continued)

	Exposure Time							
	1 hour		6 hours		24 hours		72 hours	
Trial	Control	Treated	Control	Treated	Control	Treated	Control	Treated
TREATMENT II								
1	1	0	1	0	1	0	1	0
2	1	0	1	0	1	0	1	0
3	1	0	0*	0	1	0	1	0
4	1	0	1	0	1	0	1	0
5	1	0	1	0	1	0	1	0
6	1	0	1	0	1	0	1	0
7	1	0	1	0	1	0	1	0
8	1	0	1	0	1	0	1	0
9	1	0	1	0	1	0	1	0
10	1	0	1	0	1	0	1	0
TOTALS	10	0	9	0	10	0	10	0

Table I (Continued)

	Exposure Time							
	1 hour		6 hours		24 hours		72 hours	
Trial No.	Control	Treated	Control	Treated	Control	Treated	Control	Treated
TREATMENT III								
1	Not Sampled		Not Sampled		Not Sampled		Not Sampled	
2	1	0	1	0	1	1	1	0
3	0*	0	0*	0	1	0	0*	0
4	1	0	1	0	1	0	1	1
5	1	0	1	0	1	0	1	0
6	1	0	1	0	1	0	1	0
7	1	0	1	0	1	0	1	0
8	1	0	1	1	1	0	1	0
9	1	0	1	0	1	0	1	0
10	1	0	1	0	1	1	1	0
TOTALS	8	0	8	1	9	2	8	1

Table I (Continued)

Trial No.	Exposure Time 1 hr. Control	1 hr. Treated	6 hr. Control	6 hr. Treated	24 hr. Control	24 hr. Treated	72 hr. Control	72 hr. Treated	144 hr. Control	144 hr. Treated
TREATMENT IV										
1	1	0	1	0	1	0	1	0	1	0
2	1	0	1	0	1	0	1	0	1	0
3	1	0	0*	0	1	0	1	0	1	0
4	1	0	1	0	1	0	1	0	1	0
5	1	0	1	0	1	0	1	0	1	0
6	1	0	1	0	1	0	1	0	1	0
7	1	0	1	0	1	0	1	0	1	0
8	1	0	1	0	1	0	1	0	1	0
9	1	0	1	0	1	0	1	0	1	0
10	1	0	1	0	1	0	1	0	1	0
TOTALS	10	0	9	0	10	0	10	0	10	0

*Contamination was encountered in Trial 3, causing incomplete recovery of *Vibrio fetus* from the control samples.

TABLE II

Summary of recovery of Vibrio fetus in all samples by trial, treatment, and strains used

Trial No.	Treatment I Control	Treatment I Treated	Treatment II Control	Treatment II Treated	Treatment III Control	Treatment III Treated	Treatment IV Control	Treatment IV Treated	Strain Used Rilma	Strain Used Lindy	Strain Used Vegor
	Number of times Vibrio fetus was recovered from the samples in each trial:										
1	4	0	4	0	Not Sampled		5	0	1	-	-
2	4	0	4	0	4	1	5	0	-	1	-
3*	3	0	3	0	1	0	4	0	-	-	1
4	4	0	4	0	4	1	5	0	1	-	-
5	4	0	4	0	4	0	5	0	1	-	-
6	4	0	4	0	4	0	5	0	-	1	-
7	4	0	4	0	4	0	5	0	-	-	1
8	4	0	4	0	4	1	5	0	1	-	-
9	4	0	4	0	4	0	5	0	-	1	-
10	4	0	4	0	4	1	5	0	-	-	1
TOTALS	39	0	39	0	33	4	49	0	4	3	3

1 Indicates that the strain was used in that trial.
\- Indicates that the strain was not used in that trial.

*Contamination was encountered in Trial 3, causing incomplete recovery of Vibrio fetus from the control samples.

TABLE III

Replication by treatment and exposure interval

Exposure	Treatment I		Treatment II		Treatment III		Treatment IV	
Interval	Control	Treated	Control	Treated	Control	Treated	Control	Treated
1 hr.	10	10	10	10	9	9	10	10
6 hr.	10	10	10	10	9	9	10	10
24 hr.	10	10	10	10	9	9	10	10
72 hr.	-*	-	10	10	9	9	10	10
144 hr.	-	-	-	-	--	-	10	10
30 days	10	10	-	-	-	-	-	-
TOTALS	40	40	40	40	36	36	50	50

Total replications of treated samples - 166.

Total replications of control samples - 166.

*Indicates that no observations were made at that exposure interval.

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