Further Studies on Use of Polymyxin B Sulfate with Dihydrostreptomycin and Penicillin for Control of Vibrio fetus in a Frozen Semen Process

An effective method for the destruction of *Vibrio fetus* in a frozen semen process was reported from this laboratory (4). This was obtained by the addition and thorough mixing of 1,000 units polymyxin B sulfate and 2,000 μg dihydrostreptomycin with freshly ejaculated bull semen, followed by dilution of one part semen with two parts of an egg yolk-citrate extender containing these same antibiotic levels plus 500 units penicillin, and incubation at 30°C for 30 min prior to cooling. These data are substantiated, in part, by research which has shown that the number of viable *V. fetus* organisms was reduced by dihydrostreptomycin in extended semen samples incubated at temperatures higher than 5°C, the customary temperature to which semen is cooled (1, 9).

In our hands a 30-min incubation period at 30°C reduced post-thaw motility but not fertility (4). However, an unnecessary reduction in live spermatozoa is, of course, undesirable for an efficient A.I. organization. Therefore, in an attempt to eliminate the period of incubation and resulting spermatozoan death in the procedure described above, a study was undertaken to determine whether addition of antibiotics to undiluted semen at or near body temperature immediately following collection without a period of incubation provided the necessary *V. fetus* control.

Semen-Processing Procedures

To determine the necessity of the incubation period, all semen received 1,000 units polymyxin B sulfate and 2,000 μg dihydrostreptomycin sulfate per milliliter prior to dilution. The combined antibiotics were added in distilled water at a concentration such that 0.02 ml of antibiotic solution was added per milliliter of undiluted semen. The semen and antibiotic solution were thoroughly mixed by inversion of the stoppered tube several times, and immediately thereafter one part semen was diluted in two parts of an egg yolk-citrate extender. The extender contained 1,000 units polymyxin, 2,000 μg dihydrostreptomycin, and 500 units penicillin. Following dilution, one-half of the diluted semen was incubated at 30°C for 30 min, followed by slow cooling to 5°C; the other half was similarly cooled without incubation.

At 3 hr after initial dilution, each portion received enough additional nonglycerolated extender to bring the extension ratio to one part semen in 15 parts semen-extender mixture. This extender also contained antibiotic concentrations of 1,000 units polymyxin, 2,000 μg dihydrostreptomycin, and 500 units penicillin per milliliter. Immediately thereafter, an equal portion of egg yolk-citrate extender containing glycerol and 500 units penicillin was added, bringing the final extension ratio to one part semen to 30 parts of semen-extender mixture. The final antibiotic level was, thus, 500 units polymyxin, 1,000 μg dihydrostreptomycin, and 500 units penicillin. At this point samples were taken for bacteriological examination.

The above treatments were compared to the antibiotic levels of 500 μg dihydrostreptomycin and 500 units penicillin per milliliter extended semen. These levels were recommended for use in liquid semen by Orthey and Gilman (10). The antibiotics were incorporated in the extender and the processing procedure was similar to that described above for frozen semen, except there was no addition of antibiotics to undiluted semen or incubation.

Bacteriological Procedures

One-milliliter aliquots of semen were inoculated with serial dilutions (Figure 1) of a composite of five *V. fetus* cultures. The cultures were representative of various geographical areas in
TREATMENTS:

- Initially Diluted 1 Part Semen Plus 2 Parts Extender
- Antibiotics Mixed with Undiluted Semen
- Diluted Semen Incubated

**ANTIBIOTIC LEVELS**

**TREATMENTS:**

- Initially Diluted 1 Part Semen Plus 2 Parts Extender
- Antibiotics Mixed with Undiluted Semen
- Diluted Semen Incubated

**RANGES IN NUMBER OF LIVE ORGANISMS PER ML OF UNDILUTED SEMEN x 10^6**

- 0.0030 to 0.024
- 0.038 to 0.12
- 0.30 to 0.60
- 3.0 to 3.8
- 15 to 38

**Serial Dilution of Inoculum**

**Figure 1.** Effect of addition of antibiotics to undiluted semen and incubation upon *V. fetus* growth.

* Number of colonies on filter pad.
> Greater than.
M Mold (overgrowth).
O No colonies on filter pad.

the United States and include both Type I and Subtype I organisms which correspond to Florent's *V. fetus venerealis* (2, 3). Subtype I is also known as Type III (6).

The inoculum was grown on Albinia brucella agar, using the gaseous environment of Plasteridge et al. (11). The inoculum was harvested after 72 hr by washing the organisms from the surface with saline, and the resultant suspensions were each adjusted before compositing to 10% transmission on the Evelyn photoelectric colorimeter, using a 660-μm filter. Counting of the viable organisms was performed on blood agar plates for each composite prepared. Range of those counts is shown in Figure 1.

Semen aliquots were subjected to the antibiotic treatments and dilutions as described under semen-processing procedures. Immediately after the glycerol-containing extender was added, as described above, 1-ml samples were withdrawn and cultured.

The 1-ml sample was stirred into approximately 5 ml of a 0.1% peptone solution previously placed in a Millipore 47-mm pyrex filter holder equipped with a 0.45-μm filter. After mixing, vacuum was applied. Filter pads were washed with five 20-ml portions of the 0.1% peptone solution, to insure removal of the antibiotics.

The filter pads were removed and placed on the surface of Albinia brucella agar plates (10% bovine blood added). Plates were incubated for five days under the optimum gaseous environment (11) and examined under a dissecting microscope for evidence of growth. Typical colonies were confirmed by suspending the organisms in saline and examining under the phase contrast microscope.

**Results and Discussion**

Serial dilutions of inoculum are plotted against type of antibiotic treatment in Figure 1. The first dilution tube, containing the equivalent of 15 to 38 million live vibrio organisms per milliliter of the undiluted semen, is represented at the bottom of the ordinate (Serial Dilution 1), and the fifth serial dilution, representing 3,800 to 24,000 organisms per milliliter of undiluted semen, is shown at the top (Serial Dilution 5). Each of the first two sets of bars represents five replications of one treatment. The standard method, 500 μg streptomycin and 500 units pen...
The number of colonies on each plate is indicated, within the bar, in each instance. "Vibrio fetus" recovery was essentially the same for those replicates not incubated as for those incubated. Therefore, incubation of initially diluted semen at 30°C for 30 min offers no additional control of "V. fetus" when these specific antivibrio agents are added to and mixed thoroughly with the undiluted semen immediately following collection.

Polyoxynin B sulfate and dihydrostreptomycin, mixed with freshly collected semen before cooling, provide effective "V. fetus" control. Notable are the facts that a) a warm environmental temperature accelerates the action of the antibiotics and that b) the absence of egg yolk (extender) permits maximum action against "V. fetus" by dihydrostreptomycin, whereas the presence of egg yolk has been shown to interfere with the activity of dihydrostreptomycin against "V. fetus" (1). This process has been demonstrated by cultural methods to provide control approximately the equal of that earlier reported by us and clearly superior to the antibiotic levels common to the A.I. industry (4). The inadequacy of the antibiotic levels common to the A.I. industry has been shown previously (5, 7).

It should be recalled that the standards (10) recommended by the Special Committee of the Council on Veterinary Service of the A.V.M.A., August, 1961, and adopted by the N.A.A.B., were for chilled fluid semen and were as follows: "Each cc of semen extender should contain from 500 to 1,000 µg of dihydrostreptomycin and 500 to 1,000 units of penicillin. The semen sample should be diluted at least 1:25 and remain in the treated extender at least 6 hr before use in the case of chilled, fluid semen (non-frozen)." (8) It should be noted that the Orthey-Gilman original recommendation was 500 µg and 500 units, respectively.

With a suggested range of antibiotic level—500 to 1,000 of streptomycin rather than simply 500 µg of streptomycin, and 500 to 1000 units of penicillin rather than simply 500 units of penicillin, representing changes in the 1961 revision of the 1954 code—the original Orthey-Gilman standards have generally been incompletely applied in frozen semen in the following deviating ways: a) In frozen semen, glycerol is present. Glycerol has been shown to have a profound and deleteriously limiting influence upon antibiotic activity against "V. fetus." b) In frozen semen processes, a period of six hr of antibiotic contact preglycerolization and prefreezing is not usual. c) In frozen semen, dilutions of less than 1:25 are sometimes employed.

References