STUDIES ON *VIBRIO FETUS* -- I. CONCENTRATIONS OF PENICILLIN, STREPTOMYCIN AND VARIOUS DYES NECESSARY FOR INHIBITION OF *VIBRIO FETUS* IN VITRO. II. COMPARISON OF TWO MORPHOLOGICALLY DISSIMILAR STRAINS OF *VIBRIO FETUS* BY MEANS OF ELECTRON MICROSCOPY.

by

JACK JENKS STOCKTON

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INTRODUCTION

Isolation of Vibrio fetus in pure culture from contaminated specimens is a very difficult task and attempts in a great majority of instances result in failure. Specimens submitted for bacteriological examination to determine the presence or absence of this organism are commonly contaminated. Materials such as placentas, uterine and vaginal discharges, vaginal swabs, and aborted fetuses, or organs from aborted fetuses, are those most frequently examined. It is not uncommon to observe the organism in smears made directly from the mentioned specimens. When such smears are fixed in heat and stained with crystal violet, the organism, if present, is readily seen on microscopic examination. Examination of 76 films prepared from vaginal swabs taken from cows in a herd known to be infected with V. fetus revealed 19 cows as possible carriers of this organism. Of these 19 only 2 gave V. fetus when cultured. Sixteen of the remaining 17 yielded gram-positive cocci, in clumps and in chains, and gram-negative bacilli. One specimen was bacteriologically sterile. Unless V. fetus is the only organism present in such a specimen it is very difficult to obtain a pure culture of it since other bacteria literally crowd it out.

V. fetus has been cited as a cause of retained placentas and breeding difficulties in cows, particularly in

herds free from brucellosis. No suggestion has been made as to possible therapeutic measures that might be employed to eliminate this infectious agent from the genital tract of infected animals.

The present study on the inhibitory effect of various dyes was undertaken to learn if such dyes might prove beneficial in the isolation of V. fetus from contaminated specimens. Studies with the antibiotics, penicillin and streptomycin, were carried out for the same purpose and also to learn whether or not they might offer an approach to the problem of effective treatment in cases of known infection.

The electron micrographs included in this study were made to compare, at high magnification, the morphology of two strains of V. fetus that microscopically were considerably dissimilar. The colonial characteristics likewise showed considerable difference. One strain showed distinct comma-shaped organisms typical of members of the genus Vibrio. Organisms in the other strain appeared more nearly as straight rods, the definite comma shape being absent in a great majority of individual organisms viewed.

HISTORICAL REVIEW

The earliest recording of the association of a spirillum with abortion in animals was made by Mac Fadyean and Stockman (1913) in England. The particular case recorded involved abortion in ewes. This report was not available but is mentioned in most writings dealing with the history of vibrionic infection in animals. Smith (1918) drew attention to the fact that, buried in this report, there is a brief statement that vibrio-like forms had been isolated from aborting cattle in Ireland and Wales in 1911. Mac Fadyean and Stockman named the organism Spirillum fetus.

Smith (1918) and Smith and Taylor (1919) found a spirillum, morphologically identical with that described by Mac Fadyean and Stockman, in the stomach contents of aborted bovine fetuses. This finding in the State of New Jersey was the first such infection recorded in the United States. They named the organism Vibrio fetus.

Since these two early recordings the reporting of the infection in cattle has been confined largely to the period since 1940. Reports of infection in cattle in the United States since Smith's recognition have appeared as follows: Schroeder (1920), Traum (1923) and Barger (1928) in California; Gilman (1939) in New York; Plastridge (1941) in Connecticut; Rhoades and Hardenbrook (1947) in Illinois; Webster and

Thorp (1949) in Michigan and Bell (1950) in Virginia. Plastridge and Williams (1948) have drawn attention to the probable widespread distribution of the infection in the United States as a result of tests made by them on specimens sent in by veterinarians from herds in Maine, New Hampshire, Massachusetts, Pennsylvania, Virginia, Georgia and New York. Moore (1950) stated that, to his knowledge, the disease has been found in the states of Ohio, Indiana, Wisconsin, Missouri, Kansas, Nebraska, and Oklahoma.

Vibriosis in sheep has been reported as follows: Carpenter (1920) and Baker and Stone (1939) in New York State; Welsh and Marsh (1924) in Montana; Graham and Thorp (1930) in Illinois; Ryff (1940) in Michigan; Lee and Scrivner (1941) in Wyoming, and Ryff and Lee (1945) in Wyoming.

Curtis (1913) reported the isolation of a curved motile bacillus from the uterine discharges of two women patients, one whose infection followed instrument-induced abortion. The other patient's labor, at full term, was complicated as a result of infection with this organism. Curtis assigned this rod to the genus Vibrio. Ward (1948) reported the finding of V. fetus in a pustule on the cheek of a male laboratory worker who had been engaged in research involving V. fetus for some weeks. Also isolated from this lesion was a large gram-positive, non-sporulating rod and a few short gram-negative rods. The capability of V. fetus to maintain itself within the body of human males was cited

with the possibility of the larger gram-positive rod being responsible for the small lesion and the smaller organism being restricted to the role of a secondary invader.

The need for some effective means of isolating V. fetus from contaminated specimens has been evident for a number of years. Lee and Scrivner (1941) pointed out that vaginal discharges and fetal membranes are not satisfactory for the isolation of V. fetus because of the excessive contamination invariably present. Ryff and Lee (1945) stated that, aside from Ryff's description of Hammond's use of crystal violet veal infusion agar plates in the isolation of V. fetus, no attempt has been made to develop a selective medium for the isolation of this organism although the material offered for examination is frequently such that overgrowth with contaminants will result. Hagan (1943) gave a method that has been used to isolate the organism when mixed with other bacteria, as, for example, when it occurs in a soiled placenta. He stated that after intraperitoneal inoculation of a guinea pig the organism would often survive for 3 or 4 days and might then be re-isolated from the spleen. There are no lesions evident and later the organisms disappear.

Chambers (1948) reported his experiences using penicillin in the treatment of non-breeding cows. This report gave no account of the possible causative organisms but the author classified his results as good. It has been suspected (Moore, 1950) that V. fetus might be the cause of consider-

able breeding difficulties in cattle. This author called attention to the need for some method of treating this condition. Webster and Thorp (1949) stated that V. fetus is one of the organisms that must be considered in brucella-free herds plagued with abortions, retained placentas and a low conception rate.

At the present time Difco's "Thiol" medium is the medium of choice for the cultivation of V. fetus. This is a semisolid medium. The organism can be recovered with more regularity by the inoculation of suspected material into this medium than by the inoculation of other semisolid, liquid or solid media. On occasion it has been necessary to incubate the thiol medium at 37 C for as long as 7 days before growth became evident. Bacterial contaminants, frequently found in suspected specimens, grow profusely and rapidly in this medium. Overgrowth of the vibrios occurs when such contamination is present.

MATERIALS

To determine the inhibiting powers of penicillin and streptomycin on V. fetus in vitro, these antibiotics were tested in a fluid medium and in a solid medium. No fluid medium was known that would support growth of V. fetus, and it was necessary to develop one that would satisfactorily grow this organism. Stockman (1919) described the growth of V. fetus in a broth medium, the composition of which is not mentioned. He stated that subculture from broth to broth usually failed, but, with the addition of a small portion of raw potato to the liquid medium, the culture could be kept up in series. Ryff and Lee (1945) mentioned that growth was obtained in nutrient broth, but that better development was obtained by the addition of 1.0% tryptose to Difco veal infusion medium, which contains a small percent of agar. Plastridge and Williams (1943) described a medium containing 0.3% agar in which growth occurred approximately 0.5 to 1.0 mm subsurface. This medium was considered unsatisfactory for the testing of antibiotic action since growth of the organism occurred in a limited zone only, and hence would be subjected to the inhibiting action of the antibiotics only in a small portion of the total volume. Huddleson (1948) drew attention to the excellent growth obtained by the use of Difco's "Thiol" medium. This medium contains 0.1% agar

and like the semi-solid medium of Plastridge and Williams yields growth in a limited zone, slightly sub-surface.

A number of fluid media were tried to determine if they would support sufficient growth of this organism for the purpose desired. A medium of the following composition was found to be the most satisfactory:

Chicken Infusion Broth	500 ml
Bacto-Peptone	10 gm
Distilled Water q.s.	1000 ml

The pH was adjusted to 6.8 and the medium was autoclaved at 121 C for 20 minutes.

When this medium was seeded and incubated aerobically at 37 C a slight amount of growth occurred on the original transfer from Plastridge's semi-solid medium. Subculture from this fluid medium, with incubation under aerobic conditions, was unsuccessful. When the original transfer and subsequent transfers were incubated at 37 C under 10% CO_2 all tubes consistently showed growth. Growth was faintly evident at the end of 24 hours incubation, decidedly evident at 48 hours and at 72 hours the broth was quite turbid. At the end of 96 hours a coating of the bottom of the tubes was apparent.

Blood agar plates were used as the solid medium. These were made from Difco's Blood Agar Base and 5% bovine blood.

Thiol medium supports excellent and abundant growth of V. fetus and is being used almost exclusively in attempting primary isolation of this organism. For these reasons the dye studies were performed employing this medium. By incorporation of various concentrations of a single dye into this medium the inhibitory action of several dyes for V. fetus, Micrococcus pyogenes var aureus, and Escherichia coli was studied. Stock solutions of the dyes were 1% concentrations in distilled water.

The cultures used in this study and their origin are as follows:

Vibrio fetus, Plastridge Strain. Obtained from Dr. W. N. Plastridge, Storr's Experiment Station, University of Connecticut, Storrs, Connecticut.

Vibrio fetus, Strain 490. Isolated from an aborted bovine fetus from the Michigan State College Dairy Herd, East Lansing, Michigan.

Vibrio fetus, Strain B-24. Isolated from an aborted bovine fetus submitted for bacteriological examination to the Department of Bacteriology, Michigan State College.

Micrococcus pyogenes var aureus. A coagulase positive micrococcus isolated from a lesion on the face of a man at the Department of Bacteriology, Michigan State College.

Escherichia coli. Isolated from a human stool specimen submitted to the Department of Bacteriology, Michigan State College.

The streptomycin and the penicillin used in this study were commercial lots obtained from Merck and Co., Inc., Rahway, New Jersey. The penicillin was crystalline penicillin G sodium and the streptomycin was the calcium chloride complex. Thiol medium and Bacto-Penase were obtained from the Difco Laboratories, Detroit, Michigan.

METHODS AND RESULTS

Antibiotic Studies

The fluid medium was tubed in 9.5 ml amounts. Trial had shown that autoclaving at 121 C for 20 minutes and subsequent cooling to room temperature resulted in a loss of approximately 0.25 ml of medium per tube. Solutions of the antibiotics were prepared so that the addition of 0.5 ml to the medium resulted in the unit concentration desired.

The test cultures were maintained on Plastridge's semi-solid medium. Growth from 4-day-old cultures was suspended in 4 ml of the fluid medium and vigorously shaken for fifteen minutes to assure the breaking up of bacterial clumps. An inoculum of 0.25 ml of this suspension was used to seed each tube, thus bringing the total volume in each tube to 10 ml. Counts by darkfield examination showed approximately 480,000,000 organisms per 0.25 ml of inoculum. Each concentration of the antibiotics was tested in lots of ten tubes.

Tubes were incubated at 37 C and under approximately 10% CO_2. They were observed daily for evidence of growth and were agitated at each examination. Incubation was continued for a total of 96 hours. If growth was apparent at the end of this incubation period, the contents of the tubes showing growth were examined microscopically. If growth was

not evident at the end of the incubation period, the inoculated material was transferred to thiol medium or Bacto-Penase was added to the original medium, the tubes reincubated for an additional 96 hours, at the end of which time they were again examined for growth.

Control tubes containing 9.75 ml of fluid medium were inoculated with 0.25 ml of the suspension used to inoculate the tubes containing the antibiotics. They were incubated exactly as were the test cultures.

Screening tests to obtain an approximate range of effectiveness of each antibiotic were conducted. Table 1 shows the results of these tests. Penicillin concentrations of 0.5 unit per ml and above were found to inhibit bacterial growth in all instances whereas in concentrations of 0.0125 unit per ml and lower, growth occurred in all but 2 tubes. Streptomycin in concentrations of 1.0 µg per ml and above inhibited growth in all instances whereas concentrations of 0.1 µg per ml allowed growth in all tubes. All controls showed growth.

Similar procedures were followed to determine the minimum concentration necessary for inhibition. Table 2 shows the results of these studies. Penicillin in concentrations of 0.3 unit per ml and above inhibited growth in all tubes. In a concentration of 0.2 unit per ml, 4/10 tubes of Plastridge's Strain, 2/10 of Strain 490 and 0/10 of Strain B-24 showed growth. B-24 was a recent isolate and

TABLE 1

RESULT OF SCREENING TESTS TO DETERMINE THE APPROXIMATE CONCENTRATION OF PENICILLIN AND OF STREPTOMYCIN NECESSARY TO INHIBIT THE GROWTH OF V. FETUS IN VITRO

Concentration of Antibiotic	Strain of Test Organism Used		
Penicillin u/ml	Plastridge	490	B-24
2.5	0/10[a]	0/10	0/10
0.5	0/10	0/10	0/10
0.0125	8/10	10/10	10/10
Streptomycin µg/ml[b]			
5.0	0/10	0/10	0/10
1.0	0/10	0/10	0/10
0.1	10/10	10/10	10/10
10 controls of each strain all showed growth at 96 hours.			

[a]Indicates the number of tubes showing growth over the number of tubes inoculated.

[b]The potency and dosage of streptomycin were formerly stated in "S" units, 1,000 of which correspond to 1 mg of streptomycin base. All potencies and dosages are now expressed in terms of weight of base.

did not grow as profusely in the fluid medium controls as did the other two strains.

Streptomycin showed complete inhibition in concentrations of 0.5 µg per ml. In concentrations of 0.3 µg per ml 10/10 of Plastridge's Strain grew, 9/10 of Strain 490 grew and 6/10 of Strain B-24 grew. In concentrations of 0.4 µg per ml only Plastridge's Strain grew (8/10).

TABLE 2

RESULT OF TESTS TO DETERMINE THE MINIMUM CONCENTRATION OF PENICILLIN AND OF STREPTOMYCIN NECESSARY TO INHIBIT THE GROWTH OF V. FETUS IN VITRO

Concentration of Antibiotic	Strain of Test Organism Used		
Penicillin u/ml	Plastridge	490	B-24
0.4	0/10	0/10	0/10
0.3	0/10	0/10	0/10
0.2	4/10	2/10	0/10
0.1	10/10	10/10	10/10
0.05	10/10	10/10	10/10
Streptomycin µg/ml			
0.9	0/10	0/10	0/10
0.8	0/10	0/10	0/10
0.7	0/10	0/10	0/10
0.6	0/10	0/10	0/10
0.5	0/10	0/10	0/10
0.4	8/10	0/10	0/10
0.3	10/10	9/10	6/10
0.2	10/10	10/10	10/10
10 controls of each strain all showed growth at 96 hours.			

To learn if the antibiotics tested were bactericidal or merely bacteriostatic two procedures were employed to inactivate the antibiotics following 72 hours incubation. The dilution of the antibiotic selected for trial was the mini-

mum dilution giving complete inhibition in all tubes. To half of the tubes of this dilution was added 0.1 ml of Bacto-Penase. This amount of Bacto-Penase will inactivate 1,000 units of penicillin. One hundred micrograms of streptomycin are inactivated by this quantity of Bacto-Penase. One-hundredth ml was added for the sake of convenience and to insure complete inactivation of the antibiotics. To learn if Bacto-Penase might have some inhibitory action on the test organism, 0.2 ml amounts were added to tubes containing approximately 10 ml of the fluid medium. These tubes were then seeded with the test organism exactly as were the tubes containing the various concentrations of the antibiotics. It was learned that Bacto-Penase is not inhibitory for any strain of V. fetus tested.

Inoculations were made from the remaining tubes, containing the antibiotics in minimum inhibiting concentration, into thiol medium. Thiol medium will inactivate small amounts of penicillin and streptomycin and was developed for the cultivation of specimens containing inhibiting concentrations of these two antibiotics (Huddleson, 1948).

The cultures were incubated for an additional 72 hours. Examination at the end of this period revealed that there had been no growth in any tube of minimum effective concentration of the antibiotics tested. Other concentrations, greater than the minimum inhibiting concentration, were treated in a similar manner. Tubes containing penicil-

lin showed growth in three isolated cases, one each in the following concentrations: 2.5 units per ml, 1.0 unit per ml and 0.5 unit per ml. Tubes containing streptomycin showed no growth in any instance.

The minimum concentration of penicillin and of streptomycin necessary for inhibition of V. fetus on blood agar plates was variable. For penicillin some trials showed a minimum concentration of 0.25 unit per ml as inhibitory, while other trials showed good growth at the highest concentration tested, namely, 0.4 unit per ml. Tests with streptomycin showed a variation of from 0.2 µg per ml to 0.35 µg per ml as minimum inhibitory concentrations. The highest concentration of streptomycin tested was 0.5 µg per ml. Tests using Micrococcus pyogenes var aureus and tests using Escherichia coli as seed cultures showed growth of both these organisms at all concentrations of the antibiotics tested. It appears that the use of streptomycin or penicillin in blood agar plates would not be effective in isolating V. fetus from a specimen contaminated with either the gram-positive coccus or the gram-negative rod mentioned above.

Dye Studies

One percent aqueous stock solutions were added to 100 ml amounts of thiol medium in a volume necessary to give the desired final concentration. The medium containing the dye was tubed in approximately 10 ml amounts and autoclaved at 121 C for 20 minutes. Stock cultures of V. fetus were main-

tained in thiol medium and inoculation into the dye containing medium was made by mixing the growth with the upper portion of the thiol medium and pipetting 0.1 ml amounts into the dye containing medium. The tubes were incubated aerobically at 37 C for 96 hours.

Stock cultures of M. pyogenes var aureus and of E. coli were maintained in brain heart infusion broth. Amounts of 0.1 ml from a 24 hour broth culture were transferred to the dye containing medium.

Results of these studies are recorded in Tables 3 to 7. Three of the compounds tested were found to offer no promise whatsoever as a means of inhibiting the gram-positive cocci or the gram-negative coliform organisms while allowing the vibrios to grow. Thionin, brilliant green and sodium azide inhibited growth of the three strains of V. fetus tested. However, the inhibitory concentration for V. fetus allowed both M. pyogenes var aureus and E. coli to grow abundantly. From the results obtained it is believed that two of the compounds, basic fuchsin and gentian violet, offer definite possibilities as regards inhibition of gram-positive cocci that might be present as contaminants. Basic fuchsin allowed growth of the three strains of V. fetus tested in a majority of instances in concentrations of 1 : 5,000 through 1 : 9,000, while inhibiting M. pyogenes var aureus in every instance. Gentian violet likewise showed promise since concentrations from 1 : 50,000 to

TABLE 3

EFFECT OF THIONIN ON THE GROWTH OF *V. FETUS*, *M. PYOGENES VAR AUREUS* AND *E. COLI* IN THIOL MEDIUM

Organism	Concentration of Thionin				
Strain of *V. fetus*	1-5T	1-10T	1-20T	1-30T	1-40T
Plastridge	0/2	4/4	4/4	4/4	4/4
490	0/2	4/4	4/4	4/4	4/4
B-24	0/2	4/4	4/4	4/4	4/4
M. pyogenes	2/2	4/4	4/4	4/4	4/4
E. coli	2/2	4/4	4/4	4/4	4/4

TABLE 4

EFFECT OF SODIUM AZIDE ON THE GROWTH OF *V. FETUS*, *M. PYOGENES VAR AUREUS* AND *E. COLI* IN THIOL MEDIUM

Organism	Concentration of Sodium Azide			
Strain of *V. fetus*	1-5T	1-10T	1-15T	1-20T
Plastridge	0/2	2/2	2/2	2/2
490	0/2	2/2	2/2	2/2
B-24	0/2	0/2	0/2	2/2
M. pyogenes	2/2	2/2	2/2	2/2
E. coli	2/2	2/2	2/2	2/2

TABLE 5

EFFECT OF BRILLIANT GREEN ON THE GROWTH OF *V. FETUS*, *M. PYOGENES VAR AUREUS* AND *E. COLI* IN THIOL MEDIUM

Organism	Concentration of Brilliant Green			
Strain of *V. fetus*	1-10T*	1-20T	1-30T	1-40T
Plastridge	4/4	4/4	4/4	4/4
490	4/4	4/4	4/4	4/4
B-24	4/4	4/4	4/4	4/4
M. pyogenes	4/4	4/4	4/4	4/4
E. coli	4/4	4/4	4/4	4/4

*At concentrations greater than 1-10T the medium was too dark to determine grossly if growth was present.

TABLE 6

EFFECT OF GENTIAN VIOLET ON THE GROWTH OF *V. FETUS*, *M. PYOGENES VAR AUREUS* AND *E. COLI* IN THIOL MEDIUM

Organism	Concentration of Gentian Violet					
Strain of *V. fetus*	1-50T	1-55T	1-60T	1-65T	1-70T	1-100T
Plastridge	2/6	2/6	2/6	3/6	3/6	4/4
490	3/6	4/6	5/6	5/6	6/6	4/4
B-24	3/6	4/6	4/6	5/6	6/6	4/4
M. pyogenes	0/6	0/6	0/6	0/6	0/6	4/4
E. coli	4/4	4/4	4/4	4/4	4/4	4/4

TABLE 7

EFFECT OF BASIC FUCHSIN ON THE GROWTH OF *V. FETUS*, *M. PYOGENES VAR AUREUS* AND *E. COLI* IN THIOL MEDIUM

Organism	Concentration of Basic Fuchsin						
Strain of *V. fetus*	1-5T*	1-6T	1-7T	1-8T	1-9T	1-10T	1-20T
Plastridge	4/4	4/4	4/4	4/4	4/4	4/4	4/4
490	4/4	4/4	4/4	3/4	4/4	4/4	4/4
B-24	4/4	3/4	4/4	3/3	4/4	4/4	4/4
M. pyogenes	0/4	0/4	0/4	0/4	0/4	1/4	4/4
E. coli	4/4	4/4	4/4	4/4	4/4	4/4	4/4

*At concentrations greater than 1-5T the medium was too dark to determine grossly if growth was present.

1 : 70,000 inhibited *M. pyogenes var aureus* uniformly but allowed all strains of *V. fetus* to grow in a majority of instances, with the exception of Plastridge's Strain, which showed poor growth throughout.

An attempt was made to learn the outcome of simultaneous inoculation of *V. fetus* and *M. pyogenes var aureus* into solutions of gentian violet and basic fuchsin which had shown inhibition of the gram-positive coccus but had allowed the vibrio to grow. Five dilutions of basic fuchsin and 3 dilutions of gentian violet were tried. The inoculum for each was 0.1 ml of a suspension of each organism as previously described. Only one strain of *V. fetus* (B-24) was tried, this being the most recently isolated strain. Results are recorded in Tables 8 and 9.

TABLE 8

RESULT OF SIMULTANEOUS INOCULATION OF V. FETUS AND M. PYOGENES VAR AUREUS INTO VARIOUS CONCENTRATIONS OF BASIC FUCHSIN THIOL MEDIUM

Organism	Concentration of Basic Fuchsin					
	1-5T	1-6T	1-7T	1-8T	1-9T	1-10T
V. fetus (B-24) and M. pyogenes	4/6[a]	6/6[a]	6/6[a]	6/6[b]	6/6[b]	6/6[b]

[a]Microscopic examination showed only V. fetus present.
[b]Microscopic examination showed V. fetus in abundance and an occasional clump of gram-positive cocci.

TABLE 9

RESULT OF SIMULTANEOUS INOCULATION OF V. FETUS AND M. PYOGENES VAR AUREUS INTO VARIOUS CONCENTRATIONS OF GENTIAN VIOLET THIOL MEDIUM

Organism	Concentration of Gentian Violet		
	1-50T	1-60T	1-70T
V. fetus (B-24) and M. pyogenes	0/6	4/6*	6/6*

*Microscopic examination showed only V. fetus present.

It will be seen that a dilution of 1 : 70,000 of gentian violet allowed V. fetus to grow but inhibited M. pyogenes var aureus in every instance. Basic fuchsin, in concentrations of 1 : 6,000 to 1 : 10,000, allowed V. fetus to grow in every instance with inhibition of M. pyogenes var aureus in concentrations up to 1 : 8,000. In concentrations

of 1 : 8,000 to 1 : 10,000 a few clumps of gram-positive cocci were seen on microscopic examination, but the gram-negative vibrios were present in an overwhelming majority. It was noted that, after an additional 48 hours incubation at 37 C, the growth in the tubes containing gentian violet was much more profuse than that in the tubes containing basic fuchsin.

COMPARISON BY ELECTRON MICROSCOPY OF TWO MORPHOLOGICALLY DISSIMILAR STRAINS OF VIBRIO FETUS

Rhoades and Hardenbrook (1947) were the first to publish electron micrographs of V. fetus. They attempted to show the various forms that have been reported in the literature. The pictures presented here were taken for purposes of comparison of two strains of V. fetus designated as Plastridge's Strain and Strain 490. The colonial and cellular morphology of the two strains are distinctly dissimilar.

Colonial Characteristics

Observation was made of 3 day old colonies on 5% bovine blood agar plates, incubated at 37 C in an atmosphere of approximately 10% CO_2. Examination was made using a dissection microscope and reflected light.

Plastridge Strain: The colonies were raised, moist, glistening, circular, entire and homogenous. They averaged approximately 1 mm in diameter and were nonhemolytic.

Strain 490: The colonies were raised, moist, glistening, circular, entire and slightly granular. They were decidedly smaller than the colonies of the Plastridge Strain, averaging approximately 0.5 mm in diameter. They were nonhemolytic.

Cellular Morphology

Gram stains were made of 3 day old cultures in thiol medium and were examined using oil immersion.

Plastridge Strain: The organisms were gram-negative. Numerous cells showed a densely stained granule located terminally. These granules were slightly larger in diameter than the width of the cell. Individual cells were distinctly comma-shaped and many were joined in pairs forming an S-shaped arrangement.

Strain 490: The organisms were gram-negative. Granules similar to those described in the Plastridge Strain were seen in Strain 490. Individual cells appeared slightly longer than those of the Plastridge Strain. Distinct comma-shaped cells were very few in number. A majority of the cells were straight or only slightly curved.

Merchant (1946) described the granules as appearing in both young and old cultures. Stockman (1919) reported these granules as being filterable but that no growth came from them. These granules are considerably more in evidence when the organisms are grown in thiol medium than when grown in Plastridge's semisolid medium.

The characteristics of both strains of this organism correspond to those of V. fetus as given in Bergey's Manual

of Determinative Bacteriology, 6th Edition, with the exception that the distinct comma-shape is not evident in individual cells of Strain 490. Serologically both strains agglutinate in high titer with antiserum prepared by the injection of Strain 490 into rabbits.

Specimens for electron microscopy were prepared as follows. Seventy-two hour cultures of each strain, grown in thiol medium, were suspended in 0.3% neutral formalinized saline and by differential centrifugation the cells were separated from the agar. The final preparation was a thrice washed suspension in physiological saline. Specimens were placed on collodion-covered screens and were viewed with an RCA electron microscope, Model EMU, at the laboratories of the Michigan Department of Health. The original magnification was 7,000 diameters. Magnification of the accompanying figures was arrived at by enlargement of the plates exposed at the original magnification mentioned.

Figure 1 shows the Plastridge Strain at approximately 35,000 magnifications. The individual cells have a distinct comma-shape. One S-shaped form shows a slight constriction at its middle and suggests the occurrence of fission. One individual cell shows a single polar flagellum that appears to curl and go out of view underneath the cell and then emerge on the opposite side.

Figure 2 shows a single typical cell of Strain 490 enlarged approximately 50,000 times. The distinct comma-

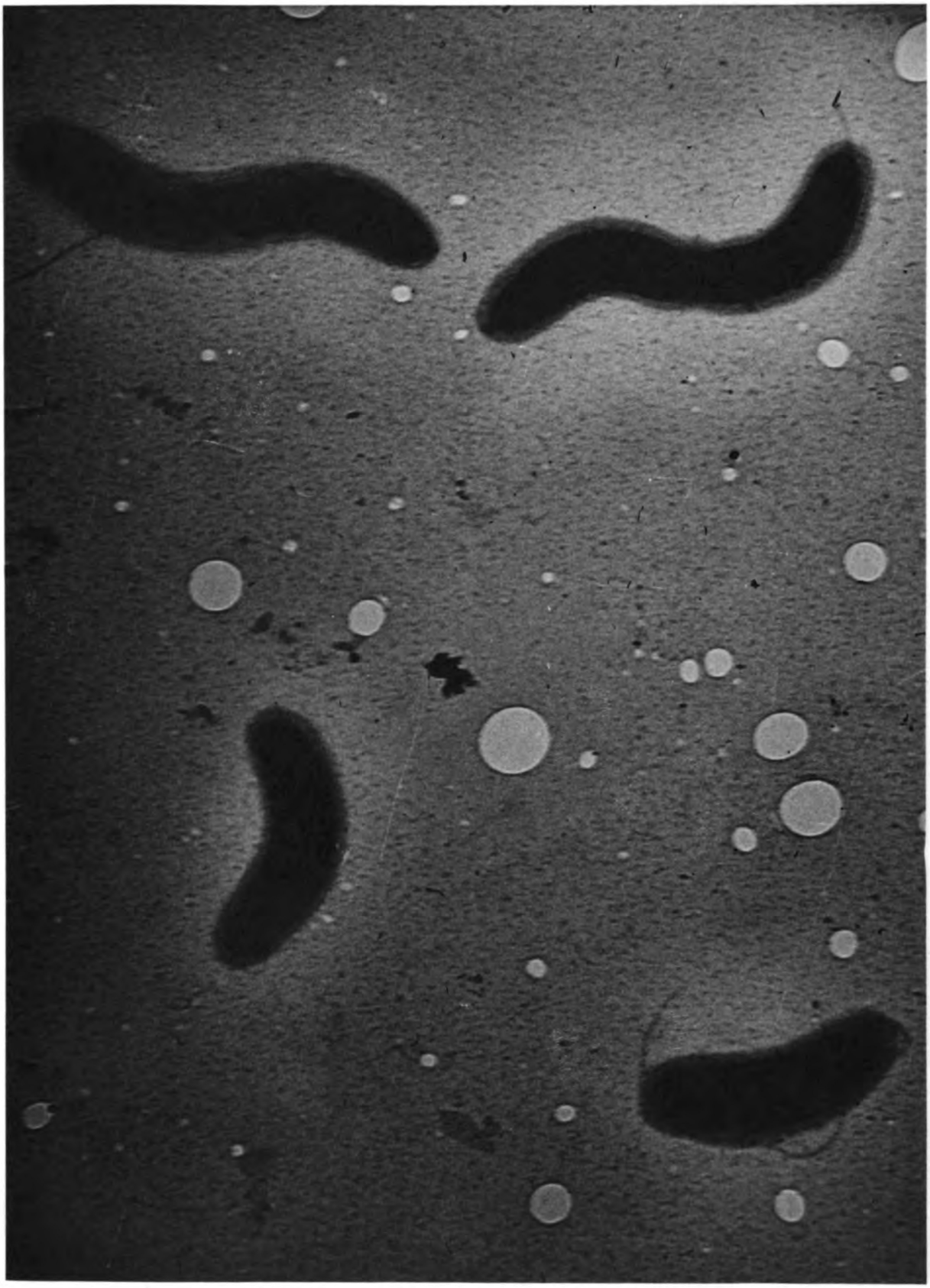

Fig. 1.--Electron micrograph of V. fetus Plastridge Strain, approximately 35,000 X.

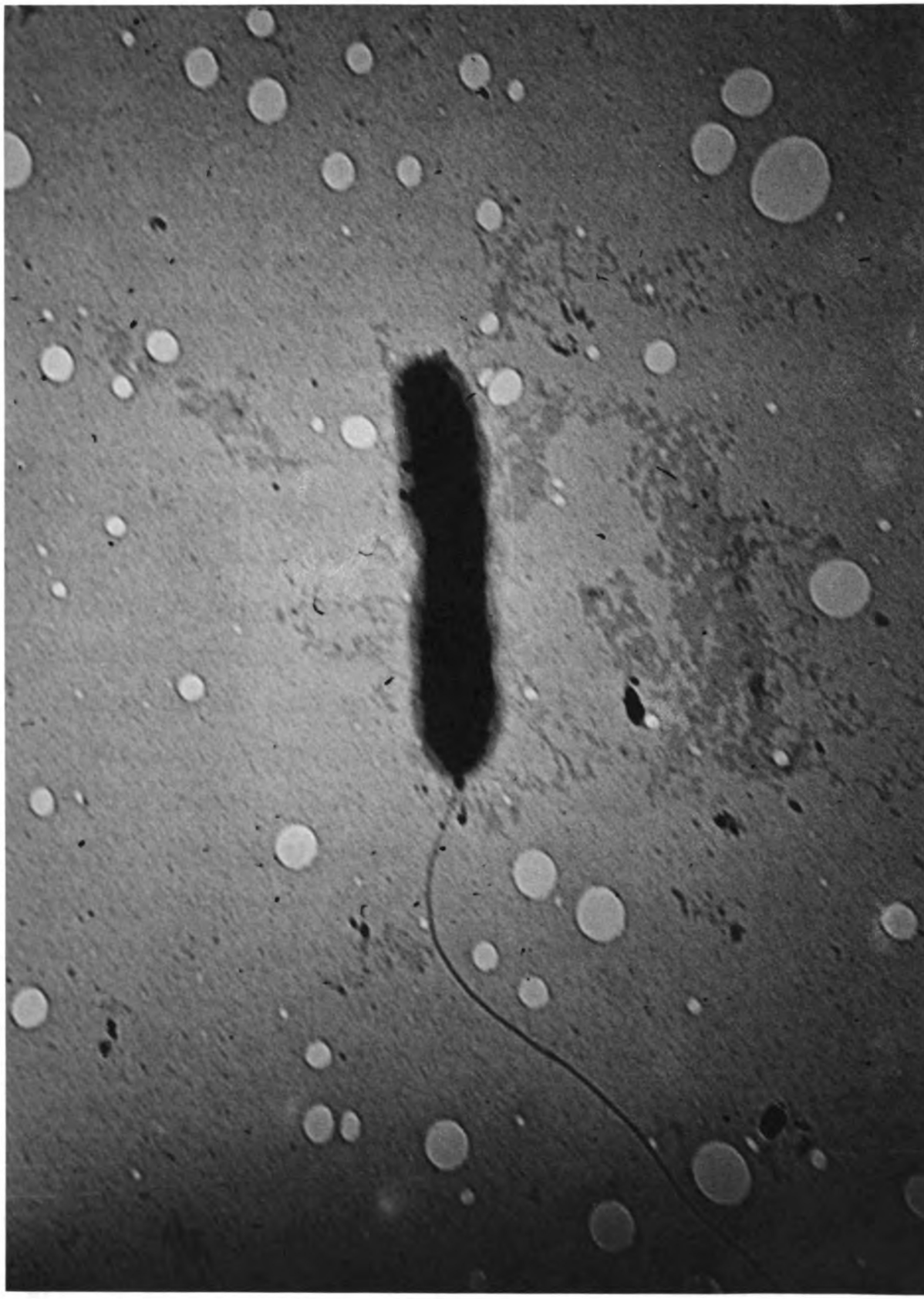

Fig. 2.--Electron micrograph of V. fetus Strain 490, approximately 50,000 X.

shape, evident in Plastridge Strain, is absent in this instance and a nearly straight rod is presented. A study of this figure with a magnifying lens shows a constriction at the middle of the cell in the grayish halo that surrounds the denser inner cell structure. A very distinct polar flagellum is seen and near the point of attachment to the body of the cell the distinct outline becomes less evident and there is a gradual blending into the material seen as a grayish halo surrounding the denser inner cell mass. It appears that the flagellum is a continuation of the outer confines of the bacterial cell. Pijper (1949), studying the flagella of Spirillum volutans by means of sunlight dark-ground microscopy, observed that the flagellum seemed to be attached to the cell wall of the organism, but that it did not pierce it. His conclusions, after study of a report by Miss van Iterson (1947) in which electron micrographs of Vibrio metschnikovii showing flagellation were present, was that in vibrios the supposed flagellum is a continuation of the cell wall, and not, as most motile bacteria, mucous twirls derived from the capsule.

Common to the organisms in both Figures 1 and 2 is the grayish halo immediately surrounding the dense inner mass of the organism. This is described by most workers as a shrinking of the cytoplasm away from the outer confines of the bacterial cell. Absent from all pictures of these two strains were the granules so noticeable on ordinary micro-

scopic examination. The small, smoothly outlined circular areas, seen in the photographs, are holes in the collodion membrane that was used as a retainer for the bacterial specimen.

DISCUSSION

The use of streptomycin or penicillin as aids in the recovery of V. fetus from a contaminated specimen does not appear to be of any practical value. The results obtained with these antibiotics incorporated into blood plates were so variable that no definite concentration could be given as the minimum inhibiting concentration for V. fetus. Representatives of the gram-positive cocci and the gram-negative bacilli, which so commonly contaminate specimens suspected of harboring V. fetus, showed growth at the greatest concentration of the antibiotics tried, whereas V. fetus was inhibited by this concentration in a majority of instances. It is felt that the fluid medium, in which the minimum inhibiting concentration of each antibiotic was determined, is not a satisfactory medium for the primary isolation of V. fetus. Thiol medium inactivates penicillin and streptomycin and was originally developed as a medium into which specimens containing inhibiting concentrations of these antibiotics could be inoculated. For these reasons it is believed that the use of penicillin and streptomycin, in attempting to recover V. fetus in pure culture from a contaminated specimen, would not be helpful.

The concentration of streptomycin necessary to inhibit growth of V. fetus in the fluid medium was approximately

0.5 µg per ml. The concentration of penicillin necessary to inhibit the growth of V. fetus in the fluid medium was approximately 0.3 unit per ml. The use of penicillin solutions for vaginal douche in non-breeding cows has been advocated by Chambers (1948). It is believed that the use of either penicillin or streptomycin in solution, given as a utero-vaginal douche, might be indicated as a therapeutic measure in cows known to be infected with V. fetus. It seems logical to suggest the use of antibiotics, known to be bactericidal in high dilution for V. fetus in vitro, when this organism is shown by examination to be an inhabitant of the bovine female genital tract.

Gentian violet in thiol medium, in a concentration of 1 : 70,000 was shown to inhibit the growth of M. pyogenes var aureus, while allowing V. fetus to grow. Basic fuchsin in thiol medium, in concentrations ranging from 1 : 5,000 to 1 : 9,000 likewise allowed V. fetus to grow but inhibited M. pyogenes var aureus. The use of either of these dye-containing media seems indicated for primary culture where the specimen is contaminated with gram-positive cocci. Presence or absence of such contamination can readily be determined by a gram-stain of the material prior to culturing. Gram-positive cocci are the most frequently found contaminants when working with vaginal swabs that have been taken in an aseptic manner.

Of the dye compounds studied none was found that

would inhibit the growth of E. coli in thiol medium while allowing V. fetus to grow. Coliforms are the most frequently found contaminants in such specimens as vaginal discharges, placentas and aborted fetuses. The finding of some agent that will hold these rapidly multiplying bacteria in check, while allowing V. fetus to grow, would make it possible to culture V. fetus from many specimens that are now utterly useless.

Electron micrographs of two morphologically dissimilar strains of V. fetus revealed considerable difference between these two strains at a magnification of approximately 35,000 diameters. Both strains were isolated from aborted fetuses and their characteristics correspond to those of V. fetus given in Bergey's Manual of Determinative Bacteriology, 6th Edition. The comma-shape so descriptive of organisms in the genus Vibrio is regularly present in the Plastridge Strain but is mostly absent in Strain 490. The flagellum of Strain 490 appears to be a continuation of the cell wall and thus is the same as that of another member of the genus Vibrio, namely V. metschnikovii (Pijper, 1949).

SUMMARY

The minimum concentration of streptomycin and of penicillin necessary for inhibition of V. fetus in vitro, using a fluid medium, was found to be 0.5 µg per ml and 0.3 unit per ml respectively.

Gentian violet in thiol medium in a concentration of 1 : 70,000 allowed the growth of three strains of V. fetus but inhibited the growth of M. pyogenes var aureus. Basic fuchsin in concentrations of 1 : 5,000 to 1 : 7,000 was found to give similar results. The amount of growth evident by gross observation was decidedly greater at the end of 6 days in the thiol tubes containing gentian violet than in those containing basic fuchsin. No dye studied inhibited E. coli while allowing V. fetus to grow.

Electron micrographs of two morphologically dissimilar strains of V. fetus showed a very marked difference in individual cellular morphology at magnifications of approximately 35,000 diameters.

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