

TECHNIQUE FOR RAPID GROWTH OF *VIBRIO FETUS* IN BROTH¹

CLAUDE V. REICH, HOWARD W. DUNNE, ALFRED L. BORTREE, AND JOHN F. HOKANSON

Department of Veterinary Science, Pennsylvania State University, University Park, Pa.

Received for publication February 18, 1957

Vibrio fetus and *Brucella abortus* are associated with infectious abortion and infertility in cattle and sheep. Vibriosis has assumed greater economic significance since brucellosis has been under better control. One of the major obstacles to the investigation of vibriosis has been the difficulty of growing the organism *in vitro* in significant quantity in liquid media. Agar must be added to increase the viscosity and this interferes with removal of the organisms for antigen evaluation.

The original isolation by McFadyean and Stockman (1913) was accomplished as a grayish subsurface film in Bang's agar-gelatin-serum medium. Smith (1918) used agar slants with added defibrinated blood to isolate *V. fetus* from aborted bovine fetuses. The tubes were sealed and growth was obtained in the syneresis fluid at the base of the slant. Welch and Marsh (1924) grew the organism on serum agar slants in sealed jars containing cultures of *Bacillus subtilis* to reduce the "oxygen tension." Plastridge (1941) described a liver infusion medium for cultivation of *V. fetus* in an environment of 10 per cent CO₂. Thiol media (Difco) was proposed by Huddleson (1948) as satisfactory for isolation and maintenance of *V. fetus*. At present, the latter, along with Albimi brucella broth plus 0.1 per cent agar in an environment of 10 per cent CO₂ are the media of choice.

With the exception of a few "trained" strains, growth is negligible on solid media in the presence of 10 per cent CO₂. Reich *et al.* (1956) reported that *V. fetus* is microaerophilic, requiring a reduced oxygen tension (pO₂ = 0.06) rather than CO₂. Good growth can be obtained on agar plates in an environment of 30 per cent air, 5 per cent CO₂, 10 per cent H₂ and 55 per cent He when incubated at 37 C. Because of the effort involved, this procedure while satisfactory for isolation and purification of culture is unsuited to the

growth of the large quantities of cells needed for antigenic complex and vaccination studies. This paper reports a procedure for growing *V. fetus* in large quantities in broth.

MATERIALS AND METHODS

Stock cultures. The cultures of *V. fetus* were maintained in screw cap culture tubes of Albimi brucella broth plus 0.1 per cent agar. They were transferred and checked for purity every 30 days.

Apparatus. A system was devised to aerate broth cultures of *V. fetus* with a gas mixture having reduced oxygen tension. This gas mixture was 35 per cent air, 5 per cent CO₂, 10 per cent H₂ and 50 per cent He. The mixture was forced into the culture through fritted glass gas dispersion tubes, after which it was collected in a reservoir from which it was recirculated. Figure 1 is a diagrammatic sketch of the system used for the gas circulation. A DeVilbiss no. 501 vacuum-pressure pump² (A) was used to force the gas mixture through a line filter (B), made of glass wool in a 4 by 50 cm glass tube, and a vapor trap (D) to a pressure-side manifold (E). This manifold had 4 stopcocks by which it was connected to the culture vessels through ground glass fittings. The culture flasks (F) were 1 L balloon flasks fitted with gas dispersion tubes and were connected with ground glass fittings on their gas collecting stoppers to a vacuum-side manifold (E') similar to that on the pressure side. The manifold-culture flask assembly was built into a 37 C incubator. The gas mixture leaving the vacuum manifold was stripped of vapor by passing into a Kjeldahl condensation bulb (G) connected to a 25 ml flask serving as a vapor trap (D). From here the gas entered a 20 L glass bottle (I) which served as a gas reservoir. This gas was recirculated to the pump through another Kjeldahl condensation bulb (G) and a 500 ml

¹ Authorized for publication on February 13, 1957, as Paper No. 2125 in the Journal series of the Pennsylvania Agricultural Experiment Station.

² The authors wish to thank The DeVilbiss Company, Somerset, Pa., for contributing technical assistance and the compressor used in this study.

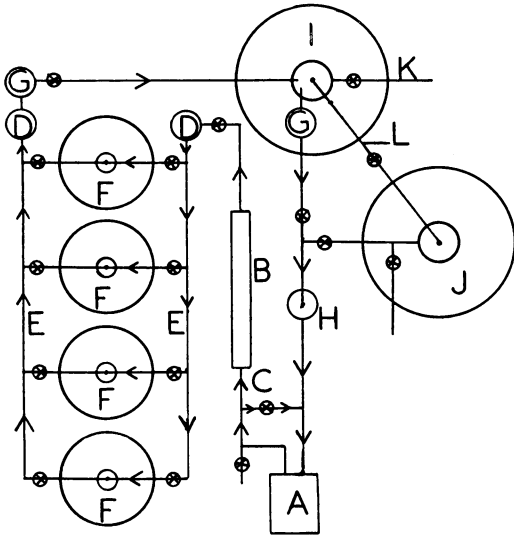


Figure 1. Schematic diagram of apparatus for cultivation of *Vibrio fetus*. A—DeVilbiss compressor no. 501. B—Line filter, glass wool. C—By-pass. D—Water traps. E—Manifolds for 4 flasks. F—Culture flasks. G—Kjeldahl condensation bulbs. H—Flask containing desiccant. I—Gas reservoir. J—Pressure stabilization bottle. K—Gas filling and sampling outlet. L—Siphon line. Arrows indicate direction of gas flow.

flask of desiccant (H). The gas reservoir was calibrated in liters and was fitted with an outlet (K) for filling and sampling. The rate of circulation of gas was controlled by a needle-valve stopcock (C) in a line by-passing the pressure and vacuum sides of the pump.

Pressure in the system was maintained at atmospheric by a 20 L glass pressure stabilization bottle (J). This was filled with water containing a fungistat and adjusted to pH 3.0 with sulfuric acid. As pressures varied inside and outside the system, this water flowed to and from the gas reservoir through a siphon line (L) to maintain equilibrium. Pressures were stabilized within the system by making the pressure line from the pump to the culture flasks of 10 mm glass tubing with 4 mm stopcocks, while the vacuum line from the flasks to the pump was made of 7.5 mm glass tubing and 2 mm stopcocks.

A line was added to connect the pressure stabilizing bottle to the vacuum side of the pump. Outlets to the atmosphere were also provided for the pressure stabilizing bottle and the pressure

side of the pump. These fittings were to facilitate filling the system.

Operation of the apparatus. The gas mixture was prepared in the gas reservoir which was first filled with the water from the pressure stabilization bottle. This was accomplished by opening the pressure side of the pump to the air and evacuating the reservoir thereby allowing the water to displace the evacuated gas until the reservoir was filled. The vacuum was then applied to the pressure stabilization bottle and the procedure reversed, except that now filtered commercial gases were admitted to the reservoir through outlet K. Air was first admitted to a level of 16 L of water remaining in the reservoir. This, plus the air in the remainder of the system, made a total of 7 L of air. Two L of hydrogen, 1 L of carbon dioxide and 10 L of helium were added in turn. At the proper level of water in the reservoir for each gas, the siphon line was closed and each gas was admitted to atmospheric pressure as recorded by a manometer on the gas reservoir.

The interiors of the culture flasks were sprayed with Dow Corning Antifoam A and filled with 600 ml of brucella broth. The dispersion tube assembly was cotton plugged and inserted into the flasks. These units were autoclaved at 121 C for 25 min. Before attaching to the manifolds, the medium was inoculated with 30 ml of 3 day old culture in semisolid broth.

Since the dispersion tubes are seldom uniform in porosity, the rate of gas flow was regulated to the slowest flask on the assembly. This was achieved by turning the by-pass stopcock (C) from the open toward the closed position. The gas flow was then equalized in the culture flasks by regulating the manifold-to-flask stopcocks on the pressure side.

Gas analysis. The gas analyses for O₂, CO₂ and H₂ were accomplished with a Burrell portable Orsat Type absorption analyzer.

Growth curves. The growth curve was determined turbidimetrically using a Spectronic 20 colorimeter with a light wavelength of 5250 Å being selected as offering the broadest reading scale. The samples of culture were taken by adapting one of the balloon flasks with a side arm opening. This opening was fitted with a rubber apron stopper. Samples were taken through the stopper with a 2.0 ml syringe and needle, at 2 hr intervals, in such manner as not to disturb the gas proportions.

RESULTS AND DISCUSSION

Comparative growth of V. fetus obtained by two methods. Figure 2 represents the growth curve of *V. fetus* obtained using the system described, as compared to the growth curve of *V. fetus* grown in semisolid broth incubated under 10 per cent CO₂ at 37 C. Both systems were inoculated with 1 per cent of a 5 day old culture of a vibrio obtained from an aborted bovine fetus. The growth density is given in per cent transmission. The curve (A) for "microaerophilic bubbling" is constructed from 11 points all falling on the curve indicated. The curve for growth in semisolid broth (B) was defined by χ -square evaluation of 6 points selected at random from the 14 values obtained during the log growth phase. The curve thus produced included all but 4 of the 14 points and these points were displaced by only ± 4 per cent. Each point used for the curves in figure 2 is the mean of the readings of triplicate cultures.

Visibly and graphically, the technique of "microaerophilic bubbling" induced an increased total yield per ml of culture medium used. The maximum turbidity of growth in semisolid medium allowed 43 per cent light transmission. This compares to a density, allowing 17 per cent

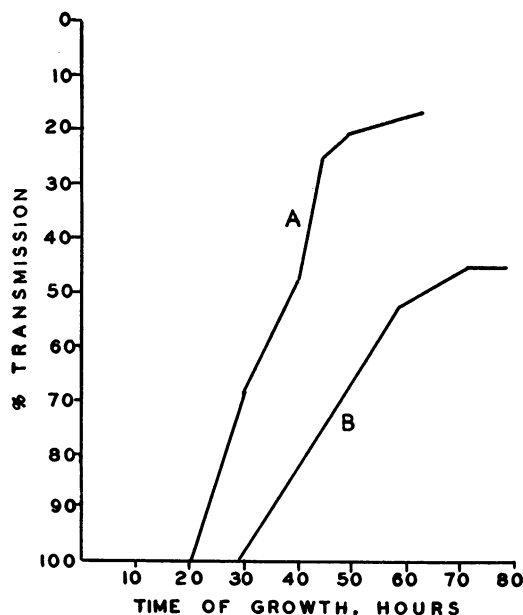


Figure 2. A. The growth curve of *Vibrio fetus* in closed gassing system. B. Growth curve of *V. fetus* in semi-solid broth.

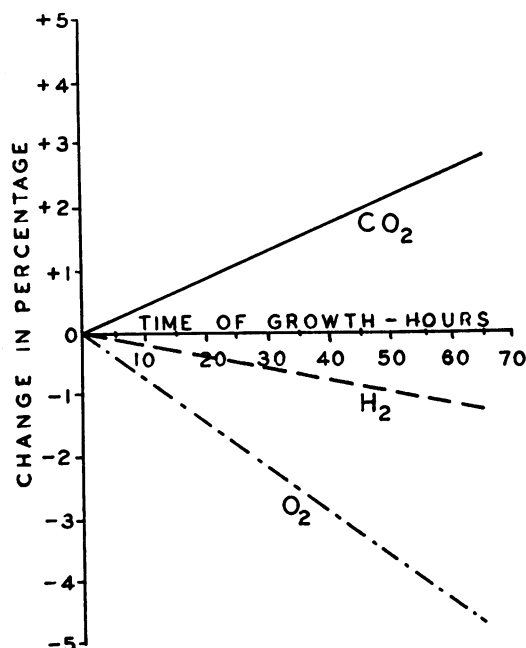


Figure 3. The change in concentration of gases in the environment during growth of *Vibrio fetus* in a closed gassing system.

transmission, obtained by the gas technique. The rate of growth was likewise increased by the procedure described, along with a reduction of 8 to 10 hr in the lag phase of growth. Curve A has a slope of 3.3 and curve B a slope of 1.4. These values indicate an increase in the rate of growth by a factor greater than 2. The absolute yield of cells for the average strain was approx. 0.25 g, dry wt, per L of culture medium. This can be reconstituted to approx 1.5 L of agglutination test antigen in saline suspension at a turbidity of 59 per cent transmission. All strains thus far grown with this technique yielded excellent antigen with respect to stability in suspension.

Interpretation of changes in gas composition: The composition of the gas mixture in the reservoir was altered by the growth of *V. fetus*. Figure 3 illustrates the general variation of the proportions of the 3 gases assumed to be active in the mixture, during the period of logarithmic growth of the organism. The values are qualitative because they proved to be dependent on the strain used, the amount and age of the inoculum, and the initial concentrations of the individual gases. The curves of the three gases were determined from one point for each gas at 65

hr. This point is the mean of 10 values determined for each gas on individual runs. In actuality, the true curves appeared to conform to the growth curve, but because of strain variation, the curves of figure 3 are given only as an indication of the trend of gas utilization by one strain.

Figure 3 shows that hydrogen and oxygen are removed from the environment and carbon dioxide is added to it. Some question could be attached to the statement of hydrogen utilization since absolute values are not involved here and there is only a deviation of -1.2 per cent in the H_2 concentration. However, it can be pointed out, that if we assume hydrogen to be metabolically inert to *V. fetus*, then in view of a decrease of oxygen in excess of the CO_2 increase, the interdependence of the ratios would dictate an increase in the percentage of hydrogen. This never happened with any strain tested. Another possibility would be that the disappearance of hydrogen is due to its solubility in the culture medium. If this were the controlling factor then two ramifications would result; the amount of hydrogen apparently utilized would be dependent on the partial pressure of hydrogen in the gas mixture and within certain minor limitations, the hydrogen concentration would remain constant after equilibration at the solubility point of the

saturated solutions. Several runs were made to test the occurrence of these events; neither occurred. Apparent hydrogen utilization was constant whether the initial concentration was 30 or 10 per cent and in neither case was there any appreciable decrease in hydrogen concentration until after the 24 hr lag phase in the growth of the organism. The organism will grow in the absence of hydrogen. However, since the gas apparently does play a minor role in the metabolic activity, it was felt that the inclusion of the gas in the mixture might serve as insurance against any alteration from its normal antigenicity.

Oxygen consumption was definite and essential to the growth of *V. fetus*, but the concentration of oxygen was critical at both extremes. Growth of most strains was inhibited when the O_2 concentration was above 10 per cent and below 3 per cent. Within 65 hr, the oxygen concentration decreased from 7 to 2.2 per cent. If the initial concentration was increased to 10 per cent, growth did occur, but the lag phase was prolonged and rapid growth generally did not occur until the concentration was reduced to approximately 8 per cent. When the concentration fell below 3 per cent, there was a noticeable decrease in the rate of growth, but growth did continue until O_2 concentration reached about 2 per cent.

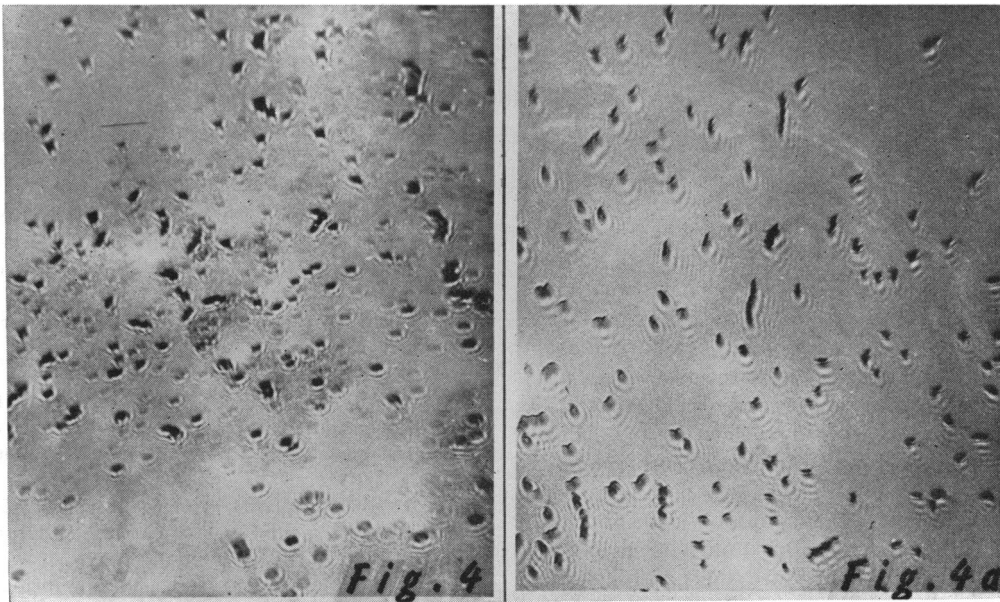


Figure 4. Photomicrograph of stained, untreated culture of *Vibrio fetus*, showing granulation. 4a. Same culture treated with 0.5% formalin before staining. Granulation has been eliminated.

Carbon dioxide was released to increase the concentration in the gas reservoir from 5 to 8 per cent in 65 hr. The over-all rise is not constant as indicated. Within 1 hr after initiating a run, the CO₂ concentration dropped from 5 to 3 per cent. It was assumed that this was due to solubility of the CO₂ in the neutral medium. After 24 hr, the concentration began to rise as indicated. If the starting concentration was adjusted from 5 to 10 per cent, the initial drop was from 10 to 7 per cent, but the final concentration went to only 12 per cent. A starting concentration of 15 per cent noticeably decreased the total cell yield. No runs were made without added CO₂, and since higher concentrations were inhibitory, the initial concentration advocated for the gas procedure was 5 per cent.

Absolute values were not used for an evaluation of this procedure because of too great a variation in the margin of error in two interdependent sets of data. An error of ± 200 ml in reading volume changes in a total volume of 20,000 ml produced insignificant values when combined with a margin of error ± 0.2 per cent in the analysis of gas ratios varying from 1 to 6 per cent from their initial values.

Variation in morphology of organism. The morphology of *V. fetus* varies from predominantly short comma and "S" forms in fresh isolates to a mixture of commas, variable length spirals, granulation and coccid forms in older stock cultures. This variation was evident during the increased growth rate induced by the gas procedure. In general, the inoculum was a culture of predominantly spirillar forms. During the later part of the lag phase, the comma and "S" forms became more evident. The early log phase was accompanied by a recurrence of the spirillar forms, and finally, the culture harvested during the late log or early stationary phases was again, with very few exceptions, completely short forms. The motility of the organism in the "bubbled" culture was markedly enhanced over those from cultures of the same age in semisolid broth.

Granulation. Stained smears of the culture showed granulation which varied in degree with the age of the culture. However, this phenomenon was not evident in wet mounts observed under the phase-contrast microscope until the mount began to dry. This suggested the possibility that the granulation was an artifact of the staining procedure. Accordingly, formalin was added to a

concentration of 0.5 per cent to a portion of a culture showing considerable granulation. After one hour, smears were made of the treated and untreated cultured material, and stained with Tunnickliff's stain for spirochetes. Figure 4a shows that formalin treatment significantly reduced the granulation observed in figure 4.

SUMMARY

A method is presented to grow *Vibrio fetus* rapidly in large numbers, in broth. The method consists of aerating broth, inoculated with *V. fetus*, with a gas mixture of 5 per cent CO₂, 35 per cent air, 10 per cent H₂ and 50 per cent He in a closed system of circulating gases.

This procedure eliminates the need for agar in the medium, increases the total cell yield, reduces the lag phase by 8 to 10 hr, and increases the rate of growth by a factor of 2. Hydrogen and oxygen concentrations were decreased and the carbon dioxide concentration was increased in the gas mixture during growth of the organism.

Variation in the morphology of the organism during growth in the "aerated" cultures has been described. Granulation ordinarily observed in stained smears of the organism was eliminated or reduced by treatment with formalin prior to preparation of the smear. This granulation may be an artifact of the staining procedure.

REFERENCES

- HUDDLESON, I. FOREST 1948 A satisfactory medium for isolation, cultivation, and maintenance of viability of *Vibrio fetus* (Bovine). *J. Bacteriol.*, **56**, 508.
- McFADYEAN, J. AND STOCKMAN, S. 1913 Great Britain Board of Agriculture and Fisheries, Report of the departmental committee on epizootic abortion. Part III. London.
- PLASTRIDGE, W. N. 1941 Cultural and serological observations on *Vibrio fetus*. *J. Bacteriol.*, **42**, 816-817.
- REICH, C. V., MORSE, E. V., AND WILSON, J. B. 1956 Gaseous requirements for growth of *Vibrio fetus*. *Am. J. Vet. Research*, **17**, 140-143.
- SMITH, T. 1918 Spirilla associated with disease of the fetal membranes in cattle. *J. Exptl. Med.*, **23**, 701-711.
- WELCH, H. AND MARSH, H. 1924 Vibrionic abortion in sheep. *J. Am. Vet. Med. Assoc.*, **65**, 203-210.