APPLIED MICROBIOLOGY, Oct. 1974, p. 730-732

Vol. 28, No. 4
Printed in U.S.A.

Tube Culture Method for Viable Counts of *Campylobacter fetus* (*Vibrio fetus*)[1]

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Received for publication 22 April 1974

A method was developed for making viable counts of *Campylobacter fetus* (*Vibrio fetus*) in tube cultures using a medium containing alkaline hematin and incubation in a carbon dioxide incubator.

Erratic or obviously inaccurate results are often obtained with viable counts of *Campylobacter fetus*. We wished to develop a procedure that would provide accurate results, permit counting of colonies with the Quebec colony counter (American Optical Corp., Cleveland, Ohio), and be practical from the standpoint of time and medium required.

In preliminary trials high colony counts were obtained on vibrio agar (VA-10) containing defibrinated bovine blood (VA-10-B), a medium we have found useful for routine isolation and culture of *C. fetus*. The formulation of this medium is: brucella broth (Pfizer Diagnostics, Clifton, N.J.), 28 g; yeast extract (Difco), 5 g; sodium succinate, 2 g; $MgCl_2 \cdot 6H_2O$, 1 g; $FeSO_4 \cdot 7H_2O$, 50 mg; agar (Difco), 20 g; and distilled water, 900 ml. After the ingredients were sterilized by autoclaving, 100 ml of defibrinated bovine blood was added and 25 ml was poured into petri plates (15 by 100 mm). Counts were reduced when smaller amounts of medium were used in the plates. A rapidly growing broth culture of *C. fetus* was diluted 10^{-6} in broth and 0.1 ml was used to inoculate the plates. The broth used had the same formulation as VA-10 without ferrous sulfate or agar. Inoculum was spread evenly over the plates with a bent glass rod. Inoculated plates were incubated for 4 days in mixed gas containing 5% oxygen, 5% carbon dioxide, and 90% nitrogen. This medium was too dark for use with the colony counter due to the blood and thickness of the medium. Elimination of the blood from the medium resulted in a reduction of the counts by a factor of 10 or more, for example, from 216 colonies to 13 colonies.

Attempts to substitute bovine blood serum or plasma for defibrinated blood were not successful, indicating that the factor essential for high counts was associated with the erythrocytes. Addition of 1% dried hemoglobin (Difco) was fairly satisfactory as a substitute for blood but offered no advantage in viewing the colonies. An alkaline hematin solution was prepared by dissolving 32 mg of hemin in 10 ml of 0.15 N NaOH and autoclaving for 30 min at 5 lb/in^2 (hemin changes to hematin in the presence of alkali). Hematin was routinely used within 3 days of preparation, but gave satisfactory results for at least 7 days if stored at room temperature. A final concentration of 0.002% hematin was obtained by adding hematin stock solution to sterile, melted VA-10 just before pouring plates. This medium was designated VA-10-H. There were no obvious differences between counts on plates of VA-10-B and on VA-10-H, indicating that alkaline hematin is a satisfactory substitute for defibrinated bovine blood in enhancing the growth of *C. fetus*.

Although *C. fetus* will readily grow on appropriate complex media without blood, it has been our observation that growth is considerably enhanced by the presence of either blood or alkaline hematin. Semisolid medium containing blood is particularly useful in recovering *C. fetus* from frozen storage. Catalase is produced by *C. fetus*, indicating the capability of synthesizing heme compounds; hematin might be useful as a precursor of heme compounds and possibly for the destruction of peroxides before catalase production in adequate amounts. Herbert (2) reported that hematin is the factor in pepsinized blood responsible for increased growth of *Yersinia pestis* on agar media, and Knisely et al. (3) used hematin in azide medium instead of sheep blood digest for growth of that organism.

The tube procedure for growth of bacteria used by Weidanz and Landy (4) for bactericidal assay of natural antibodies was modified for use in viable counts of *C. fetus*. Eight milliliters of melted VA-10-H (1.5% agar instead of 2.0%) at

[1] Contribution from Agricultural Experiment Station, Montana State University, Bozeman, and published as Journal series no. 500.

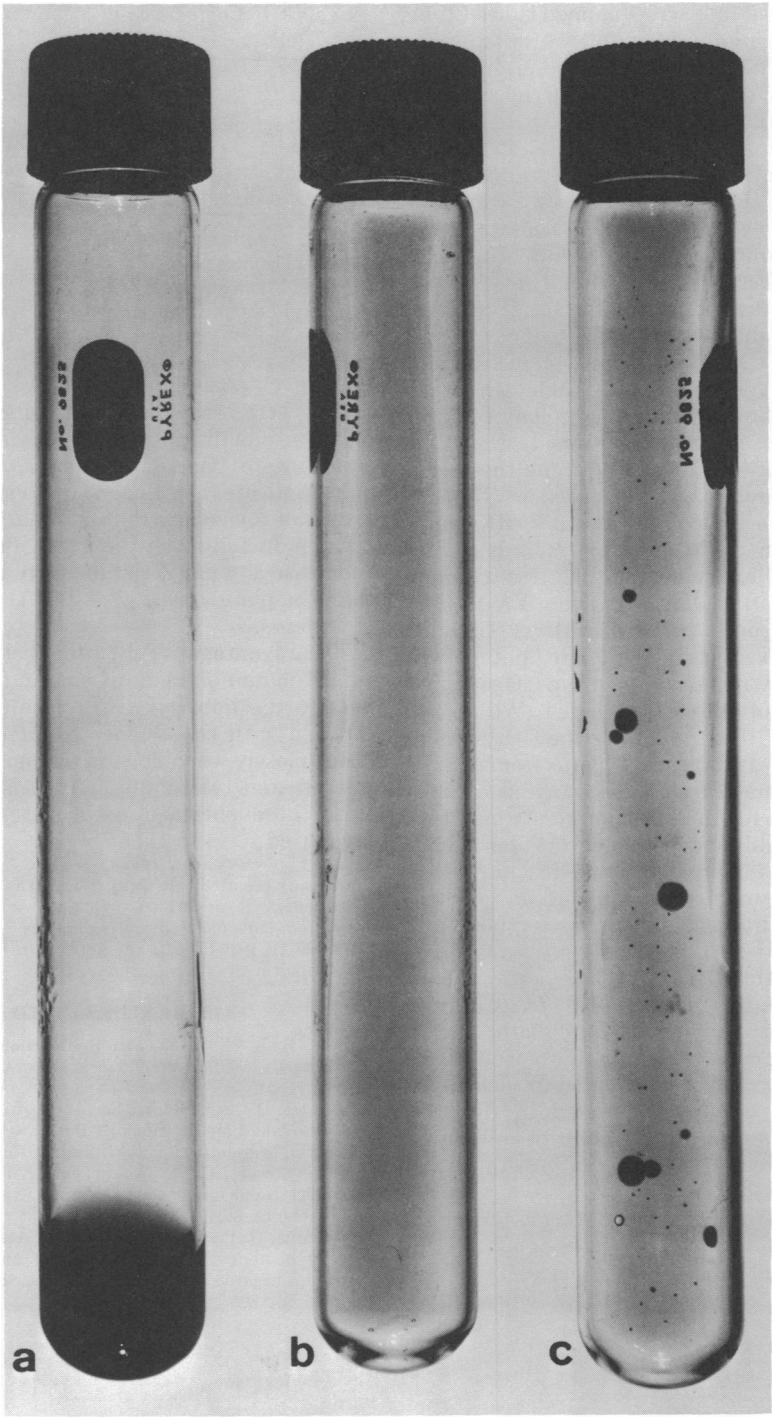

FIG. 1. *Tubes (25 by 200 mm) containing (a) 8 ml of unslanted agar, (b) 8 ml of slanted agar, and (c) C. fetus colonies after 5 days of incubation in the slanted agar.*

48 C was poured into screw-capped tubes (25 by 200 mm) containing 1 ml of a 10^{-7} broth dilution of *C. fetus*. The agar and broth were mixed by gently swirling and slanted to yield the maximal surface area of agar, allowed to harden, and incubated for 5 days in 5% CO_2 in a CO_2 incubator (National CO_2 Incubator, National Appliance Co., Inc., Rochester, N.Y.) with caps loose. Good growth of colonies was obtained and they were readily counted by holding the tubes over the Quebec colony counter (Fig. 1). The small colonies are under the surface of the agar but clearly visible, whereas the surface colonies are large but do not conceal any colonies imbedded in the agar underneath them when the tubes are held over the Quebec colony counter. Equivalent results were obtained when the tubes were sealed in jars containing the mixed gas, but inasmuch as it was necessary to incubate them in a horizontal position with consequent problems in keeping the jar lids tightly closed the CO_2 incubator was found more convenient. Petri plates containing VA-10-H or VA-10-B supported good growth of *C. fetus* under the mixed-gas atmosphere, but growth was essentially nonexistent in the CO_2 incubator for some of the isolants tried. We do not recommend at this time using the CO_2 incubator for primary isolation of *C. fetus* from clinical materials. Clinical specimens, such as cervical mucus where growth of contaminants may be a problem, should be cultured on blood or hematin agar plates incubated under the previously described gas mixture. We have had good results with culturing blood in commercially available blood culture bottles containing Trypticase soy broth (BBL) and CO_2.

We compared the growth of *C. fetus* in tube assays incubated in the CO_2 incubator and in the regular incubator in air. Two isolants of each *C. fetus* group were assayed in quadruplicate. All the isolants incubated in the CO_2 incubator produced good growth of individual colonies, whereas there was no growth in the tubes incubated in air.

A comparison was made of colony counts obtained with VA-10-H in tubes incubated in the CO_2 incubator and on petri plates incubated under the mixed-gas atmosphere. Six isolants each of *C. fetus* groups A-1, A-sub-1, A-2, B, and C were assayed (1). All tube assays were done in quadruplicate (or more) and plate assays were done in duplicate, triplicate, or quadruplicate. An analysis of variance of the means of the colony counts by the two methods was conducted using the F test (Table 1). The tube assays yielded significantly higher counts than the plate assays ($P < 0.001$) for all groups of *C. fetus*.

TABLE 1. *Comparison of colony counts by two methods*

Method	No. of observations	Mean colony count ($\times 10^7$) ± SD[a]	Analysis of variance		
			DF[b]	MS[c]	F value[d]
Tube	98	229 ± 12.76	1	425,800	37.184
Plate	91	134 ± 8.51		11,450	

[a] SD, Standard deviation.
[b] DF, Degrees of freedom.
[c] MS, Mean square.
[d] Level of significance $P < 0.001$.

The advantages of the tube method are: (i) a small amount of medium is used, (ii) assays can be set up quickly, (iii) colonies are easily counted with the Quebec counter, (iv) counts check closely when done in triplicate or quadruplicate, and (v) counts are consistently higher than those obtained with plate methods we have used.

The work reported here was performed as a contribution to the Western Regional Research Project W-112 and was supported in part by Cooperative Agreement no. 12-14-100-11, 189 (45) with the Agriculture Research Service, U.S. Department of Agriculture.

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