# QUALITATIVE AND QUANTITATIVE STUDIES OF VIBRIO FETUS ANTIGEN PRODUCED IN VARIOUS MEDIA, WITH EMPHASIS ON THE EFFICACY OF A FLUID MEDIUM

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# INTRODUCTION

The etiological role of Vibrio fetus in abortions of cattle and sheep has necessitated improved methods of diagnosis and control of this organism. The use of semi-solid media has facilitated the isolation and cultivation of the organism, but the fastidious growth requirements of V. fetus have hindered the development of an efficient method of antigen preparation. It is desirable, for antigen preparation, that the organism of choice give a good yield of cells per volume of medium and the antigen be easily prepared. Semi-solid media do not lend themselves well to the latter of these criteria.

Preparation of V. fetus antigen from a semi-solid medium requires tedious centrifugation and washing to separate cells from agar. The amount of work and time required make this method impractical for antigen preparation. It is, therefore, advantageous either to use a medium which contains no agar or one which is sufficiently solid to permit ready removal of the organisms from its surface.

Since V. fetus requires an oxygen tension intermediate between aerobic and anaerobic conditions, a large inoculum, and from three to four days incubation for perceptible growth, it is necessary to employ a medium which will permit maximum growth under these conditions.

The following experiments were carried out for the purpose of comparing solid, semi-solid and broth media for cultivation of V. fetus for the

preparation of a satisfactory antigen. Special consideration was given to the most efficient method of securing a high yield of cells. An attempt was made to prove the usefulness of a broth as a satisfactory medium for antigen production. The agglutination reaction, with known negative and positive sera, was used to determine the agglutinating titer of each antigen as influenced by the medium in which it was cultivated.

## HISTORICAL REVIEW

The first isolation of V. fetus was reported by McFadyean and Stockman (1913). Stockman in a later publication (1919) stated that the disease was first discovered in sheep, and was commonest in this species. Theobald Smith (1918) established V. fetus as the causative agent in several bovine abortions in which Brucella abortus could not be found. He named the organism Vibrio fetus.

The occurrence of vibrionic abortions in sheep was reported by Mattick (1925), Welch and Marsh (1924), Graham and Thorp (1930), Lee and Scrivner (1943) and Ryff (1940). Bovine abortions were reported by Schroeder (1920), Barger (1927), and more recently by Plastridge and Williams (1943), Rhoades and Hardenbrook (1947) and Bell (1950). These authors reported the disease to be widespread, having been found principally in New York, Illinois, Michigan, California, Montana, Wyoming, and Connecticut.

In early cultural studies, Stockman (1919) obtained a good initial growth in ordinary broth media, but was unable to successfully cultivate the organisms by subsequent transfer. However, he was able to maintain cultures by adding a small amount of potato to the broth tubes. Growth was rarely observed on nutrient agar slants, but if the agar was seeded while it was still liquid and then quickly solidified, growth occurred just beneath the surface of the agar. He stated that blood agar was very satisfactory for surface growth on slants or plates, but for antigen preparation cultures in potato broth were best.

Smith and Taylor (1919) found that growth was readily obtained from sealed nutrient agar slants provided that a few drops of defibrinated horse blood had been added to the water of condensation. Growth appeared only on the circumference of the agar near the water of condensation and gradually spread in a fine white film between the agar and the tube after 2 to 3 days incubation at 37° C. Growth did not occur in nutrient gelatin or beef peptone bouillion, but did occur in both media when a few drops of defibrinated blood were added.

Welch and Marsh (1924) obtained results similar to those of Smith and Taylor by using serum agar slants in sealed tubes. They also used a brain medium in liver broth which had been used in their laboratory for routine anaerobic cultivation. Good growth was obtained in 24 hours. Rhoades and Hardenbrook (1947) used blood agar and tryptose crystal-violet agar plates in an atmosphere of 10 per

cent $CO_2$ for initial cultures, but found subcultures to die out after one or more transfers. Bell (1950) isolated V. fetus by inoculating blood agar plates and semi-solid beef heart infusion broth (0.3 per cent agar) with the stomach contents of an aborted fetus. Growth on both media was observed after 3 days incubation at 37° C. in an atmosphere of 10 per cent $CO_2$. Subcultures were established in the semi-solid heart infusion agar but not on the blood agar. Lee and Scrivner (1941) successfully used cystine veal-infusion broth in sealed tubes for cultivation of V. fetus.

Ryff and Lee (1945) in studies on various cultural media, stated that Difco veal infusion with added salt and agar was superior to nutrient agar, tryptose agar, Difco tryptose glucose agar and Difco brain veal agar. However, the addition of blood to these media stimulated growth equal to that in the veal infusion. Their studies on hydrogen ion concentration with Difco semi-solid veal infusion agar revealed optimum growth at about pH 7.0 with lesser growth at both pH 6.0 and pH 8.0.

After several unsuccessful attempts to grow cells for antigen on solid media and in agar-free broth, Plastridge and Williams (1943) found it necessary to use a semi-solid liver-infusion agar (0.3 per cent agar). The antigen was prepared by differential centrifugation to remove the agar. The cells were washed and then suspended in a small amount of 0.3 per cent formalinized saline. For use in the tube agglutination test, the cells were diluted with

formalinized saline to a density of 1.5 on the McFarland nephelometer scale.

Huddleson (1948) stated that Difco "Thiol medium" was satisfactory for the isolation and maintenance of V. fetus. Plastridge, Williams, and Roman (1949) reported that isolation and cultivation of V. fetus has been facilitated by the use of a semi-solid medium, but it is difficult and expensive to prepare satisfactory agglutination antigens from a semi-solid medium. They suggested the use of Difco Thiol medium (0.1 per cent agar) with the addition of 2.5 per cent agar. The antigen was prepared by washing the culture from the agar surface with a small volume of 0.3 per cent formalinized saline.

## MATERIALS AND METHODS

For cultural purposes a strain of V. fetus was obtained from Dr. W. N. Plastridge, of Storrs Agricultural Experiment Station, University of Connecticut, Storrs. Stock cultures were maintained in Thiol medium, with subcultures being made every two or three weeks.

The following media used for antigen production are listed below:

1. "Thiol medium", Difco

| | |
|---|---|
| Bacto proteose peptone #3 | 10 gm. |
| Bacto yeast extract | 5 gm. |
| Bacto dextrose | 1 gm. |
| Sodium chloride | 5 gm. |
| Thiol complex | 8 gm. |
| Bacto agar | 1 gm. |
| p-amino benzoic acid | 0.05 gm. |

Thirty grams of dehydrated powder was dissolved in 1000 ml. of distilled water, adjusted to pH 6.8 and tubed in 10 ml. portions. The medium was sterilized in the autoclave for 20 minutes at 121° C. (15 lb. pressure).

2. "Thiol Broth", Difco

This medium, supplied by Difco for experimental purposes, contains the same ingredients as "Thiol medium" except agar. The medium was prepared in a manner similar to that employed for Thiol medium using 29 grams of dehydrated powder for each 1000 ml. of distilled water.

3. Solid Thiol Agar (used as slants)

This medium was recommended by Plastridge for the preparation of agglutination antigen. The medium was prepared by dissolving 25 grams of agar

and 0.05 gm. glutathione in 1000 ml. of "Thiol medium". After sterilization in the autoclave, the agar was allowed to harden in a slanting position.

4. Chicken Infusion Broth

The chicken broth was prepared by adding one liter of distilled water to each two pounds of lean chicken meat. It was placed in the steamer for three to four hours, and the resulting broth decanted from the cooked meat. The broth was allowed to cool so the fat present would solidify, and was filtered through filter paper. This gave a double strength infusion broth which was diluted with equal parts of distilled water before use.

Ten grams of Bacto peptone was dissolved in the chicken broth, and the medium adjusted to pH 6.8, tubed in 10 ml. portions and sterilized in the autoclave for 20 minutes at 121° C.

5. Chicken Infusion semi-solid medium

This medium was prepared from chicken infusion broth, with the addition of 3 grams of agar for each 1000 ml. of medium. Plastridge and Williams described the successful use of a similar medium containing beef liver broth instead of chicken broth.

6. Chicken Infusion Broth with added glutathione

To 1000 ml. of chicken infusion broth 0.05 gram of glutathione was added before sterilization. Glutathione, a reducing agent, was added to remove part of the dissolved oxygen in the liquid for the purpose of providing a more satisfactory culture medium for V. fetus. The medium must be used shortly after sterilization before the glutathione is oxidized.

In making subcultures of V. fetus from stock cultures of semi-solid medium it was necessary to suspend the organisms in broth. For this purpose brain heart infusion broth, Difco, was prepared. The medium was tubed in 5 ml. portions and sterilized in the autoclave for 15 minutes at 121° C. Suspensions of V. fetus were prepared by placing 4 ml. of brain heart infusion over the agar surface of each stock culture and the sub-surface growth suspended by agitating the broth with a pipette.

## PROCEDURE FOR THE PREPARATION OF VIBRIO FETUS ANTIGEN

1. "Thiol Medium"

Seed cultures were prepared by transferring 0.25 ml. of brain heart infusion suspension from stock cultures to a

series of 10 tubes containing 10 ml. each of Thiol medium. The tubes were incubated aerobically at 37° C. for 3 days, and sub-cultures from this series were made and incubated in the same manner. The third transfer was made into 100 tubes of medium. One seed tube was used for each 10 tubes inoculated. After incubating at 37° C. for 3 days, growth was found within the first 0.5 cm. from the surface of the medium. The organisms were removed by a water aspirator into a large Erlenmeyer flask, and treated with 0.3 per cent formalinized saline.

The suspension was centrifuged at 1600 r.p.m. for 30 minutes to remove the agar by sedimentation and the cell-containing supernatant decanted. The agar was resuspended in formalinized saline and again centrifuged. The supernatant fluids were combined and centrifuged at 4500 to 5000 r.p.m. for 60 minutes in an International Equipment Company PR1 refrigerated centrifuge. The cells were washed with formalinized saline and re-centrifuged. The sediment of cells was then diluted with formalinized saline to 100 ml. and stored at 4° C.

2. Thiol Broth

The procedure for the preparation of antigen from Thiol broth was similar to that described under Thiol medium. Preliminary tests showed the minimum inoculum required to sustain growth to be 0.5 ml. for each transfer. The initial inoculation was with a brain-heart-infusion suspension from stock

cultures and subsequent cultures could be made directly from the broth medium. Elimination of agar also permitted the direct removal of cells from the broth by centrifugation. A black precipitate which was present in the medium after incubation, was removed by filtering the antigen through absorbent cotton.

3. Solid Thiol Agar Slants

Seed tubes were prepared by transferring four 4-mm loopfuls of stock culture to solid thiol agar slants and spreading each loopful over an area of 1-2 sq. cm. After incubating at 37° C. for 4 days in an atmosphere of 10 per cent $CO_2$, the colonies were spread over a larger area and re-incubated. Sub-cultures were made from these seed tubes to 100 slants for antigen production. The cells were washed from the surface with a small volume of formalinized saline, and the suspension filtered through absorbent cotton. The antigen was then washed by centrifugation as described under Thiol medium and diluted to a volume of 100 ml.

4. Chicken Infusion Broth

The method of preparing antigen from this medium was the same as used for Thiol broth. Preliminary tests showed the minimum required inoculum to be 0.5 ml. per 10 ml. of broth when cultured in an atmosphere of 10 per cent $CO_2$ at 37° C.

Cultures could be initiated aerobically with a 1.0 ml. inoculum, but died out suddenly in the following transfer.

5. Chicken Infusion Semi-solid Agar Medium

The preparation of antigen from this semi-solid medium was described under Thiol medium, with the exception that all cultures were incubated in an atmosphere of 10 per cent $CO_2$ at 37° C.

6. Chicken Infusion Broth with added glutathione

The method of preparation was the same as for chicken infusion broth. It was found that a heavy black precipitate formed in the broth tubes similar to that in Thiol broth. Since this sediment did not form in ordinary chicken infusion broth, it was concluded that its presence was due to glutathione. The precipitate was easily removed by filtering the cell suspension through absorbent cotton.

Standardization of Antigen

The six antigen suspensions from each medium were prepared in 100 tubes containing 10 ml. per tube. As mentioned before, the volume of cells for each preparation was diluted to 100 ml. This was an arbitrary volume, but it did give a basis for determining the dilution factor required to give a density equal to that of the standard antigen. This standard antigen had a density corresponding to 1.5 on the McFarland Nephelometer scale and was supplied by Dr. W. N. Plastridge.

All density determinations of the antigen were made with the Beckman Model B Spectrophotometer using a light wave of 650 mu (red) and a slit opening of 0.82 mm. The instrument was adjusted to 100 per cent transmission of light with formalinized saline as a blank. After the instrument was adjusted, slight variations in current were observed. Consequently, it was necessary to reset the blank reading after determination of the density of each antigen.

Before density readings of the antigens could be taken, all suspensions had to be shaken vigorously with a few glass beads for 15 minutes to break up clumps of cells. The standard antigen was found to give 76 per cent transmitted light or an optical density of 0.118. Each concentrated antigen was diluted to give the same per cent transmission of light as the standard antigen. Dilutions were made by adding 0.01 ml. portions of concentrated antigen from a serological pipette to 1.8 ml. of formalinized saline. The suspension was thoroughly mixed after each addition of antigen and readings were taken until the desired density was reached.

Agglutination tests

For determination of the agglutination titers of each antigen, 4 serum samples were supplied by Dr. J. J. Stockton, Department of Bacteriology and Public Health, Michigan State College. In previous tests, Sera Nos. 1332 and 1298 were positive while sera Nos. 1250 and 1326 were negative in the tube agglutination test for V. fetus infection.

The sera were diluted from 1-50 through 1-3200 by placing 0.08 ml. of serum in a tube containing 4 ml. of the respective standardized antigen and making subsequent serial two-fold dilutions in tubes containing 2 ml. of the antigen. The control tube contained 2 ml. of antigen.

All tubes were incubated at 37° C. and readings taken at 24 and 48 hours. The readings were the same at both intervals. Complete agglutination was designated as 4, no agglutination as 0, and partial agglutination as 3, 2, or 1, depending on the degree of agglutination.

## RESULTS AND DISCUSSION

It was found in the case of Thiol medium and chicken infusion semi-solid agar that a 0.25 ml. inoculum of broth suspension was sufficient to sustain growth in 10 ml. of the media. For the three broth media, however, a minimum inoculum of 0.5 ml. was required. It was noted that growth in a semi-solid medium was concentrated near the surface while in broth the growth was dispersed throughout the liquid. These observations suggest two factors responsible for growth of V. fetus in artificial media:

1. The inoculum should be of sufficient volume to initiate satisfactory growth in the respective media.

2. Conditions for growth in a broth medium are favorable only at a depth in the liquid where the oxygen tension is intermediate between aerobic and anaerobic conditions.

It was observed that colonies on solid Thiol agar slants would develop only where the inoculum contained large numbers of organisms. When each loopful of organisms was spread over the surface, care had to be taken not to streak them over too large an area.

The black precipitate formed during the incubation of Thiol broth and glutathione infusion broth cultures was believed to be due to the presence of sulfur in the Thiol broth and in the glutathione infusion broth.

Table 1 shows the dilution required for each antigen to give an optical density equal to that of the standard antigen, and the total volume of standardized antigen obtained from each 100 ml. of concentrated cell suspension. The standard antigen was found to give 76 per cent transmitted light or an optical density of 0.118. When each of the 100 ml. volumes of concentrated antigen had been diluted to give the same density as the standard antigen, it was found that solid Thiol slants and chicken infusion semi-solid medium gave the largest volumes, 2040 ml. and 2000 ml. respectively. Thiol medium yielded 1900 ml. while Thiol broth, chicken infusion broth

Table 1

Standardization of V. fetus Antigen and a Comparison of the Total Volume from Various Media*

| Culture Medium | Dilution of Concentrated Antigen to Equal Density of Standard Antigen** | Total Volume (ml.) of Standardized Antigen*** |
|---|---|---|
| Thiol Medium | 1.00-18 | 1900 |
| Thiol Broth | 1.35-18 | 1670 |
| Solid Thiol Slants | 0.95-18 | 2040 |
| Chicken Inf. Broth | 0.93-18 | 1440 |
| Chicken Inf. Semi-Solid | 1.15-18 | 2000 |
| Glutathione Chicken Inf. Broth | 1.42-18 | 1370 |

* Determinations made with Beckman Model B Spectrophotometer with wave length 650 mu and slit opening of 0.82 mm.

** Standard antigen gave 76 per cent transmitted light or optical density of 0.118.

*** This volume represents the total volume obtained from each 100 cc. of concentrated antigen suspension.

and glutathione chicken infusion broth yielded 1670, 1440 and 1370 ml. of antigen respectively.

These results would indicate that solid and semi-solid media were superior to broth media in supporting growth of V. fetus, but the amount of work and time required in preparing antigens from the semi-solid media must also be considered. When a semi-solid medium was used, two or more additional periods of centrifugation were required to separate the agar from the cells. When solid Thiol slants were used it was found that loop transfers took considerably more time than pipette transfers.

Agglutination Tests

Table 2 shows agglutination titers for each antigen with positive serum No. 1332. Antigen from Thiol broth, chicken infusion broth, and glutathione chicken infusion broth gave a titer of 1-400 while antigen from Thiol medium, chicken infusion semi-solid, and solid Thiol slants gave titers of 1-200, 1-200 and 1-100 respectively. Table 3 supports the results of Table 2 by showing that consistently high titers were obtained with all antigens from broth media while antigens from agar media gave titers about 1 or 2 two-fold dilutions lower.

Tables 4 and 5 show agglutination titers with two negative sera Nos. 1250 and 1326. Antigen from Thiol broth and glutathione chicken infusion broth gave a non-specific titer of 1-100 while antigen from

Table 2

Efficacy of Various Media for Antigen Production Indicated by Agglutination Titers (Serum 1332)

| Source of Antigen | Serum - Antigen Dilution | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1-50 | 1-100 | 1-200 | 1-400 | 1-800 | 1-1600 | 1-3200 | Antigen Control |
| Thiol Medium | 4 | 4 | 4 | 2 | 1 | 0 | 0 | 0 |
| Thiol Broth | 4 | 4 | 4 | 4 | 2 | 0 | 0 | 0 |
| Solid Thiol Slants | 4 | 4 | 2 | 1 | 0 | 0 | 0 | 0 |
| Chicken Infusion Broth | 4 | 4 | 4 | 4 | 2 | 0 | 0 | 0 |
| Chicken Infusion Semi-Solid | 4 | 4 | 4 | 1 | 0 | 0 | 0 | 0 |
| Glutathione Chicken Infusion Broth | 4 | 4 | 4 | 1 | 0 | 0 | 0 | 0 |

Table 3

Efficacy of Various Media for Antigen Production Indicated by Agglutination Titers (Serum 1298)

| Source of Antigen | Serum - Antigen Dilution | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1-50 | 1-100 | 1-200 | 1-400 | 1-800 | 1-1600 | 1-3200 | Antigen Control |
| Thiol Medium | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 |
| Thiol Broth | 4 | 4 | 4 | 4 | 1 | 0 | 0 | 0 |
| Solid Thiol Slants | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Chicken Infusion Broth | 4 | 4 | 4 | 1 | 0 | 0 | 0 | 0 |
| Chicken Infusion Semi-Solid | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Glutathione Chicken Infusion Broth | 4 | 4 | 4 | 4 | 2 | 0 | 0 | 0 |

Table 4

Efficacy of Various Media for Antigen
Production Indicated by Agglutination Titers
(Serum 1250)

| Source of Antigen | Serum - Antigen Dilution | | | | | | | Antigen Control |
|---|---|---|---|---|---|---|---|---|
| | 1-50 | 1-100 | 1-200 | 1-400 | 1-800 | 1-1600 | 1-3200 | |
| Thiol Medium | 4 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Thiol Broth | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Solid Thiol Slants | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chicken Infusion Broth | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chicken Infusion Semi-Solid | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glutathione Chicken Infusion Broth | 4 | 4 | 2 | 1 | 0 | 0 | 0 | 0 |

Table 5

Efficacy of Various Media for Antigen Production Indicated by Agglutination Titers (Serum 1326)

| Source of Antigen | Serum - Antigen Dilution | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1-50 | 1-100 | 1-200 | 1-400 | 1-800 | 1-1600 | 1-3200 | Antigen Control |
| Thiol Medium | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thiol Broth | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Solid Thiol Slants | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chicken Infusion Broth | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chicken Infusion Semi-solid | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glutathione Chicken Infusion Broth | 4 | 4 | 1 | 0 | 0 | 0 | 0 | 0 |

the other media gave a 1-50 non-specific titer. The only exception to this was encountered with the antigen from Thiol medium which did not give a non-specific titer with serum No. 1326.

Indications are that the three broth media were superior to the agar media with respect to agglutination titers. Even though broth media were not as suitable as agar media for producing large quantities of antigen the time and work saved by using a broth medium compensated for the differences in antigen volume obtained.

## SUMMARY

Six media were studied as to suitability for *V. fetus* antigen production. The total volume of standardized antigen obtained per volume of medium and the agglutination titers of each antigen were determined. Consideration was given to the time and work required to prepare each antigen.

Solid Thiol agar slants, Thiol medium and chicken infusion semi-solid agar were superior to broth media in supporting growth of *V. fetus*, but antigen from broth cultures was more easily prepared. Agglutination titers were higher in the antigens made from *V. fetus* grown in broth media.

It is suggested that *V. fetus* antigen for agglutination tests be prepared from cultures in Thiol broth or chicken infusion broth according to the method described in this paper.

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