TESTICULAR INFUSION AGAR—A STERILIZABLE CULTURE MEDIUM FOR THE GONOCOCCUS

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INTRODUCTION

The use of testicular infusion agar suggested by Hirschfelder (1914) aroused the hope that a medium for the cultivation of the gonococcus had been found which might be sterilized by steam, thus avoiding the addition of raw albumin (ascitic fluid or blood) to agar with its uncertain sterility and frequent failure to support growth even when sterile. Unfortunately I cannot agree that by the use of his formula all difficulties in cultivating the gonococcus (at least in pure culture) are removed, as he claims. I have however, determined *some* of the factors affecting successsful cultivation of gonococci in comparatively large quantities upon a sterilizable agar containing infusion of testicle.

Vannod (1905) claimed that proper adjustment of the reaction with sodium carbonate facilitated cultivation of the gonococcus on so called ordinary media but the possible variation from the optimum is so slight that the method has not come into general use. One of Vannod's later contributions (1907) testifies to the general acceptance of the idea of the necessity of adding raw albumins.

More recently some success in improving the media has been attained by Schwarz and McNeil (1912) in this country with so called "salt free" veal agar, which is now generally used in the preparation of polyvalent antigens for the alexin fixation test.

[1] The experimental work of this paper was carried out and its practical application made in The Cutter Laboratories, Berkeley, California, and it is published with the consent of the Director, Dr. H. E. Foster.

Abroad, Lumiere and Chevrotier (1913) have advocated a mixture of beer wort and albumin sterilized in the autoclave, to which however, the addition of sterile horse serum is said to be advantageous, though not indispensable. Upon this medium gonococcus cultures were found to be viable at remarkably low temperatures (Lumiere and Chevrotier, 1914).

Emile Weil and Noire (1913) have also suggested an agar containing whey, peptone, saccharose and urea. I have failed in several attempts to corroborate their claim that gonococci would grow upon this medium.

I have not tried the cultivation of gonococci upon the egg broth of Besredka and Jupille (1913) nor according to the method of Ohlmacher (1915) upon Loeffler's blood serum but in several tests upon the starch agar of Vedder (1915) I have found it to be one of the most promising media. However, the growth, while possibly less long lived upon testicular infusion agar, is so much more abundant that the use of the latter is recommended for the preparation of gonococcic vaccine. It should prove equally valuable for the preparation of antigens to be used in the alexin fixation test but this remains to be determined.

CULTURES

My strains of gonococci came originally from clinically typical cases of urethritis and epididymitis, having been isolated upon blood agar and cultivated in some cases as long as two years on ascitic agar. All were typical gram negative biscuit shaped diplococci, showing sparse growth upon rabbit blood or ascitic agar and failure of growth, at least in the second subculture, upon plain agar at 37°C. These have been our criteria and while we have had a realization quickened by the work of Broughton-Alcock (1914) that we might occasionally exclude true gonococci thereby we have not hesitated to insist that our media should be tested particularly on the less saprophytic strains. At the conclusion of each experiment therefore the purity of each culture was checked by gram stain and failure of growth upon plain agar at 37°C.

Torrey's strains "C," "K," "L," "N," "O," and "S" kindly supplied by Dr. Charles Krumwiede, Jr., of the New York City Board of Health, conform to the above requirements and like our own were found to grow abundantly upon testicular infusion agar.

The hope that opportunity might be found for making comparative tests of this medium in the isolation of gonococci from lesions has already deferred publication so long that it seems likely this will have to be left for some one more advantageously situated. Dr. H. E. Foster,[2] has however succeeded sufficiently often in cultivating the gonococcus from cases of gonorrhea to warrant making such comparisons.

EXPERIMENTAL WORK

Successful cultures were first secured with a slightly acid medium comprising aqueous infusion of beef testicle (500 grams per litre of distilled water), 2 per cent Witte's peptone, 2 per cent agar, 0.5 per cent glucose, and 0.3 per cent NaH_2PO_4, nearly neutralized with N/1 NaOH and sterilized by intermittent steaming in the Arnold sterilizer on three successive days for 30 minutes at 100°C.

This formula differs from Hirschfelder's particularly in its sugar content, the advisability of which was shown by Elser and Huntoon (1909) and by Martin (1911). In each succeeding series of experiments a single factor was varied, the control consisting in a combination previously found successful; as the limits of variability of each factor involved were determined the preferable procedure was adopted for the following experiments.

Thus it was quickly shown that sterilization in the autoclave at 10 pounds pressure for 30 minutes is permissible. The substitution of veal for testicle infusion was found to yield a less vigorous growth. The optimum amount of testicle was found to be 500 grams per liter although fair growth resulted when the proportion was as low as 125 grams per liter of water. The

[2] Personal communication.

use of equal parts of veal extract seemed not to decrease the volume of growth but we have adhered to the use of testicle infusion alone.

AGAR

The amount of agar is important as shown in table 1. Media were made from the same testicular infusion in four lots with 1, 2, 3 and 4 per cent agar. After sterilization the slanted tubes were left in the incubator at 37°C. for three days to dry out

TABLE 1

Optimum amount of agar

AGAR %	CULTURE	16 HOURS	40 HOURS*	64 HOURS
1	G2	None	Poor	Slight
	G3	None	None	None
	G5	None	None	None
2	G2	None	Fair	Fair
	G3	None	Fair	Fair
	G5	Slight	Fair	Good
3	G2	Slight	Fair	Good
	G3	Slight	Fair	Good
	G5	Slight	Fair	Good
4	G2	Slight	Fair	Fair
	G3	Slight	None	Excellent
	G5	None	None	Good

* Patchy colonies respread on all tubes.

NOTE. In this and other tables a "slight" growth approximates that of *B. influenzae* upon blood agar; a "fair" growth corresponds to that of *B. typhi* upon plain agar; "good" to that of *B. coli;* and "excellent" to that of *Bact. pneumoneae.*

the surface of the slopes, a point shown to be necessary by ample experience. The slopes were inoculated from 48 hours ascitic agar cultures, incubated at 37°C. and observed daily.

Soft testicular infusion agar has in our hands regularly yielded less satisfactory results than that which was more firm and less moist, a fact apparently at variance with the experience of McCann (1896) working with cyst fluid agar, and Van Saun (1913) with "salt free" veal agar. Firm testicular infusion agar is moreover not only favorable for growth but facilitates

the removal of the gonococci without the admixture of solid particles of medium.

Warden (1915) has recently pointed out that one of the factors in the autolysis of gonococci is excessive moisture; it is suggested also that weak acids may inhibit autolytic disintegration. At any rate smears from testicular infusion agar cultures contain more whole cocci than those from ascitic agar, but whether the acid reaction due to fermentation of the glucose or the freedom of the media from excessive moisture, or both, may be held responsible in this case can not be stated with certainty. However, the factor of moisture in the media had to be reckoned with in all our experimental and practical work so that frequently where a clear cut result could not be obtained with fresh media there was little difficulty after a few days drying.

I have chosen 3 per cent agar as the most suitable for further use.

GLUCOSE

In the preparation of a portion of one lot of medium the usual glucose was omitted. The prepared slants were dried at 37°C. for 48 hours and afterwards at room temperature for four days. The media still appeared quite moist and the growths upon the controls where they appeared at all were patchy and unsatisfactory even on further incubation after respreading. Further drying at room temperature for ten days however led to a satisfactory result as shown in table 2, cultures being made from 24 hour ascitic agar slants and incubated at 37°C.

TABLE 2

Omission of glucose

MEDIA	CULTURE	16 HOURS	40 HOURS	60 HOURS
With added glucose, 0.5 per cent	G1	Good	Excellent	Excellent
	G2	Good	Excellent	Excellent
	G3	Good	Excellent	Excellent
Without added glucose.....	G1	None	Slight	Slight
	G2	Fair	Good	Good
	G3	Fair	Fair	Good

The usual control tests failed to show contamination in any tube so that the above result demonstrates that growth is possible without added glucose but is not so good as with it.

That a modicum of carbohydrate is necessary is indicated by a lot of media made as usual with the exception of added glucose and from which only a portion[3] of the tissue carbohydrates had been removed by the growth of *B. communior*, in which no growth of gonococci could be secured. But media which had been so fermented and then re-inforced by addition of 1 to 2 per cent glucose yielded very excellent growth showing that inhibition in the fermented media could scarcely have been due to the accumulation of metabolic wastes from *B. communior*. Thus it was shown that for these strains the order of preference for added glucose content in media previously fermented by *B. communior* is 1 per cent, 2 per cent, and 3 per cent. Since, however, I have found no advantage in a preliminary fermentation the addition of 0.5 per cent glucose to unfermented media has been retained.

PHOSPHATES

The use of unsaturated phosphates in culture media for bacteria was recommended by Henderson and Webster (1907) for their stabilizing effect upon the reaction, and a medium of this sort plus human serum was advocated by Martin (1911) for the cultivation of the gonococcus.

My experiments upon the *necessity* of *added* phosphate have been inconclusive; at times excellent growth has been secured without its addition. In three separate double lots of testicular infusion agar made with and without the addition of inorganic phosphate the advantage has been in favor of that containing it. I have made no effort to determine the optimum amount or to attempt the cultivation of gonococcus in phosphate free media.

[3] That the tissue sugar was not completely eliminated was proven by further gas production in deep tubes of the supposedly sugar free testicular infusion agar by *B. communior*.

REACTION

The inclusion of 0.3 per cent NaH_2PO_4 permits a considerable variation in the amount of sodium hydroxide added to reduce the titrable acidity. The results of a typical controlled experiment upon this point are shown in table 3. I might mention here having previously encountered some difficulty in the addition of

TABLE 3

Range of reaction of testicular infusion agar

CUBIC CENTIMETERS N/1 Na OH ADDED IN 110 cc.	TITRE		CULTURE	16 HOURS	40 HOURS	64 HOURS
	Hypothetical end point*	Actual end point				
0	+6.0	-6.0	G1	None	None	None
			G3	None	None	None
			G5	None	None	None
2	+4.0	+4.2	G1	None	None	Slight
			G3	Slight	Fair	Good
			G5	Slight	Fair	Good
4	+2.0	+3.2	G1	Fair	Excellent	Excellent
			G3	Fair	Excellent	Excellent
			G5	Good	Excellent	Excellent
6	0	+1.7	G1	Good	Excellent	Excellent
			G3	Fair	Excellent	Excellent
			G5	Good	Excellent	Excellent
2	−2.0	?†	G1	Slight	Good	Excellent
			G3	Slight	Good	Excellent
			G5	Fair	Good	Excellent
10	−4.0	Alkaline †	G1	None	None	None
			G3	None	None	None
			G5	None	None	None

*Assuming no unsaturated compounds.

† Media darkened by caramelization—end point uncertain.

more than sufficient alkali to saturate the phosphate, the glucose being thereby caramelized on heating with resultant inhibition of growth of the gonococcus.

A liter of medium was prepared with the usual testicular infusion, 2 per cent peptone, 3 per cent agar, 0.5 per cent glucose and 0.3 per cent Na H_2PO_4. Before neutralization 5 cc. titrated hot with phenolphthalein required 6 cc. N/20 NaOH to show

color. Six lots of 100 cc. each were separated and to each was added the amount of N/1 NaOH shown in table 3, and the total volume of each lot was then equalized at 110 cc. by addition of distilled water. The various media were tubed, sterilized in the autoclave at 10 pounds for 30 minutes, slanted, and dried at 37° C. for six days. Inoculation was made from 24 hour testicular infusion agar cultures; incubation was at 37°C. and the usual control tests confirmed the purity of the growth observed.

It may be seen that "excellent" results may be expected between the limits of actual titre to phenolphthalein from below +1.7 to +3.2 Normal acidity. There will be found little if any difficulty in the reaction when adjustment is made by addition of N/1 NaOH as if the acidity were to be reduced to a titre of zero. It will still be found sufficiently acid, thanks to the phosphate, to obviate the difficulty of caramelization.

PEPTONE

The recent scarcity of Witte's peptone has necessitated experiments upon the substitution of an American product. These have shown "Difco" peptone of the American Digestive Ferments Co., Detroit, Michigan, to be equal to Witte's for this purpose.

VIABILITY

In contrast with the experience of Vedder (1915) with starch agar, prolonged viability cannot be claimed for cultures of gonococci upon testicular infusion agar. It was found that daily transfer of several strains for two weeks was eminently successful; planting every other day failed to keep some of the strains alive for more than four transfers and in all of these there was evidence of deterioration.

Inoculating testicular infusion agar as well as ascitic agar, starch agar, "salt free" veal agar or blood agar from another medium we have often found it necessary to coax the growth by repeated transfers and especially by respreading, before the maximum crop could be obtained. For this reason much emphasis should be placed upon the importance of personal experi-

ence in handling gonococcus cultures. I am pleased to thank Miss Vera Bennett who has prepared much of the culture media for me and Miss Lettie Watkins who has assisted in keeping the stock cultures alive and planting the experimental media.

SUMMARY

The formula now followed in preparing testicular infusion agar for the growth of the gonococcus in preparing suitable vaccines.

1. Mix 500 grams ground beef testicle from which the tunica vaginalis has first been stripped, with 1000 cc. distilled water.
2. Soak overnight at room temperature.
3. Heat to 50°C. Keep warm for one hour by placing in the incubator at 37°C.
4. Boil, strain, and restore to 1000 cc. with distilled water. If in excess do not reduce by boiling since overheating is injurious.
5. Add 2 per cent peptone (Witte's or Difco), 3 per cent agar chopped fine, 0.5 per cent glucose, 0.3 per cent NaH_2PO_4.
6. Soak at least one hour to soften the agar.
7. Melt in the autoclave at 10 pounds pressure for 30 minutes.
8. Titrate with phenolphthalein and add N/1 NaOH sufficient to neutralize if no unsaturated compounds were present.
9. Check the titre by repetition. 5 cc. should require from 1.0 to 2.0 cc. N/20 NaOH to display color hot.
10. Tube and sterilize in the autoclave at 10 pounds for 30 minutes.
11. Slant or pour into plates.

This medium may be melted for plating, etc., but the less heating the better. Filtration for the purpose of removing the distinct turbidity of the medium also seems to be a disadvantage.

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