

DEVELOPMENT AND EVALUATION OF  
A SIMPLE CHEMICALLY DEFINED MINIMAL GROWTH MEDIUM  
FOR THE DETECTION AND IDENTIFICATION OF  
NEISSERIA GONORRHOEAE

Submitted in Partial Fulfillment of the Requirements for  
Graduation with Honors to the Department of Biology at  
Carroll College, Helena, Montana

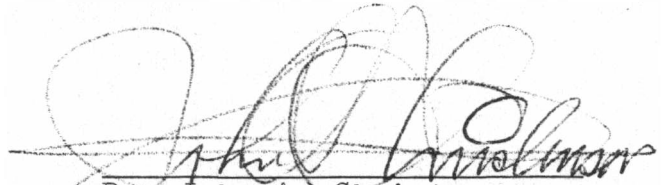
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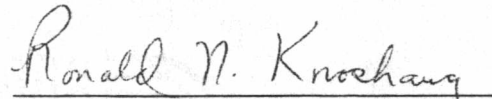



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## ABSTRACT

A simple chemically defined medium was developed and shown to be useful in the detection of nutritional mutants of Neisseria gonorrhoeae. These nutritional mutants are most often problematic organisms found in gonococcal infections. The procedure used in preparation of the medium will enable researchers to easily produce a specific medium in a relatively short time. Variations of the medium can be achieved through simple alterations in the composition of one of three stock solutions used.

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## INTRODUCTION

Problematic organisms of Neisseria gonorrhoeae are often found to be nutritional mutants (9). These organisms are typically difficult to grow, identify, and type according to their nutritional requirements. Past studies done on the development of a chemically defined medium for growth of Neisseria gonorrhoeae have shown techniques to be laborious, time consuming, and insufficient in sustaining growth of nutritional mutants. Procedures are inefficient for making alterations in the composition of the media for detection of different mutants.

The purpose of this research was to design a chemically defined medium which could be made in a short time, with minimum effort and few complications. It must be possible to store the medium and have it remain stable. The medium must be sufficient to sustain growth of nutritional mutants of Neisseria gonorrhoeae. The medium should be easily alterable in its composition to facilitate detection and differentiation of mutants.

## LITERATURE REVIEW

### Etiology

Neisseria gonorrhoeae are spherical or oval gram negative cocci, often seen in pairs with flattened sides. They do not form capsules or spores. The gonococci have two plasmids of different sizes present normally, and show five morphologically distinct forms of colonies. They require high humidity and CO<sub>2</sub>, a temperature of 35-36° C and a pH of 7.2-7.6 for normal growth. Neisseria gonorrhoeae are fastidious organisms, unable to utilize maltose, sucrose or lactose. They are strict aerobes and hence do not ferment glucose by the Embden-Meyerhof-Parnas pathway. Glucose, lactate and pyruvate can be used for energy.

Many gonococcal strains show nutritional peculiarities. Such peculiarities are the basis for auxotyping. Isolates from different geographic areas frequently differ in nutritional requirements. A synthetic, totally defined culture medium is necessary for auxotyping strains (5, 13).

### Diagnosis

Most screening tests for laboratory diagnosis of gonorrhoea involve seeding a plate of modified Thayer Martin medium (MTM) and performing an oxidase test (7, 10). Gram stains are used for samples of urethral discharge from infected males, conjunctival discharge, and purulent synovial fluid. These will reveal polymorphonuclear

leukocytes and intracellular gram negative diplococci. Serological tests on infected males have shown an immunological response of a considerable degree. Humoral antibody of the IgG classes can usually be detected. IgA can be detected early in urethral secretions of infected males. Lymphocyte blastogenesis and cell mediated immunity are activated. In females, the immunofluorescent antibody technique and the antipilus antibody determination techniques have been most useful clinically. However, experience with those tests is limited (13). Cultural examination is more sensitive than microscopic examination of smears unless organisms are nonviable, unusually sensitive to inhibitors in selective media, or the growth - supporting ability of the medium is poor. A small percentage of gonococci are sensitive to the vancomycin in MTM, but will grow on MTM without antibiotics or similar nonselective enriched media such as blood chocolate agar (7).

#### Diseases

Gonococci colonize mucosal surfaces lined by transitional or columnar epithelium, such as those of the urethra, uterine cervix, anal canal, throat, and conjunctiva. Mucosal infection usually causes inflammation and symptoms but is sometimes associated with little or none (3). Inflammation usually subsides eventually even without treatment, but the host may still be a carrier and a potential source of infection for months afterward.

In some cases, the gonococcus spreads by direct local invasion or through the bloodstream to produce major complications. In women, local spread can infect the fallopian tubes, causing salpingitis; or Bartholin's glands, causing bartholinitis. A major complication in women is pelvic inflammatory disease (PID), with two serious consequences. PID causes sterility because of the scars from the infection. The scars block the passage of ova down the fallopian tubes. Scar formation also blocks the normal flow of fluid through the fallopian tubes. Where liquid accumulates, infection by other bacteria, often anaerobic, may develop. This complication leads to chronic PID (7, 13). In men, epididymitis and periurethral abscesses occasionally result. Men can also develop urethral strictures and prostatitis. At infancy, the disease is generally contracted while going through the infected birth canal. Most often this affects the eyes and results in blindness to the child. Now, however, this is preventable with the use of silver nitrate treatments at birth. Disseminated gonococcal infection is usually distinguished by arthritis and cutaneous lesions. Endocarditis, meningitis, hepatitis, and myocarditis also result in some cases (6,7, 13).

#### Background

Research into nutritional requirements of Neisseria gonorrhoeae has determined that variations are limited

according to geographical locations (11). In 1973, Catlin and Carifo designed a chemically defined medium for N. gonorrhoeae (1, 2). The medium has had many difficulties associated with it. The Catlin medium and modified Catlin media have failed to sustain growth of some isolates and have proven to be difficult in their preparation (4, 8, 11, 12). In 1980, Wong, Shockley, and Johnston attempted to modify a medium which would support the growth of clinical and laboratory - adapted isolates of gonorrhoeae while still being chemically defined. They used 135 clinical isolates and six American Type Culture Collection reference strains. Their work resulted in the auxotyping of 135 strains of N. gonorrhoeae. Of these isolates, 44% were prototrophic, 20.7% showed proline auxotrophy, 13.3% showed arginine auxotrophy, 3.7% required methionine, and 3.0% required serine for growth (11).

## MATERIALS AND METHODS

### Media

The initial medium used was the Wong-Shockley-Johnston medium (WSJM). The composition for WSJM is shown in Table 1. Cysteine, isoleucine, valine, uracil, and tryptophan were dissolved in 0.5 N NaOH; aspartic acid and tyrosine were dissolved in 1.25 N NaOH; cystine, glutamine, leucine, phenylalanine, and hypoxanthine were dissolved in 0.3 N HCl. Biotin was dissolved in 50% (vol/vol) ethanol. All other ingredients were soluble in water.

Using the WSJM as a base medium composition, alterations were made as follows: Different lots of medium were made using glucose concentrations of 0.75%, 1.0%, 1.5%, and 2.0%. Trials were made with the concentration of solution II at 0.1%, 0.5%, 1.0%, 2.0%, and 5.0%. Agar was added to different lots in concentrations of 1.0%, 2.0%, and 5.0%.

Stock solutions were first organized separating Solution I, Solution II, Solution III, amino acids, and additional compounds. Components were sterilized, before all were combined, according to whether they required autoclaving or filter-sterilizing.

The medium was then made dividing stock solutions into three parts, according to sterilization requirements. Part A, containing Solution I, MgCl<sub>2</sub>, Agar, and glucose, was autoclaved at 121° C for 15 min, and stored at 4° C. Part B, containing Solution III, all amino acids, and all

TABLE 1. Composition of  
Wong-Shockley-Johnston medium (WSJM) (11).

Compounds	Final Conc (mM)
Solution I	
$K_2HPO_4$	23.0
$KH_2PO_4$	7.4
NaCl	85.5
Sodium acetate	25.5
Solution II	
Nicotinamide adenine dinucleotide	.003
Thiamine hydrochloride	.006
Thiamine pyrophosphate	.004
Calcium pantothenate	.0084
Solution III	
Sodium lactate	1.3
Glycerol	10.0
Oxaloacetic acid	1.52
Additional Compounds	
Hypoxanthine	0.024
Uracil	0.071
Glutathione	0.15
Biotin	0.004
Glucose	42.0
$Fe(NO_3)_3$	0.01
$MgCl_2$	8.8

TABLE 1. (cont.)

Compounds	Final Conc (mM)
Amino acids	
L-Alanine	1.12
L-Arginine-hydrochloride	0.71
L-Asparagine	0.17
L-Aspartic acid	3.76
L-Cysteine-hydrochloride-water	0.35
L-Cystine	0.15
L-Glutamic acid	8.83
L-Glutamine	0.34
Glycine	0.33
L-Histidine	0.10
L-Isoleucine	0.23
L-Leucine	0.69
L-Lysine-hydrochloride	0.27
L-Methionine	0.10
L-Phenylalanine	0.15
L-Proline	0.43
L-Serine	0.48
L-Threonine	0.42
L-Tryptophan	0.39
L-Tyrosine	0.39
L-Valine	0.51

additional components except glucose and  $MgCl_2$ , was filter-sterilized and stored at  $4^{\circ}C$ . Part C contained Solution II and was filter-sterilized and stored at  $-30^{\circ}C$ .

To make 1 liter of complete medium, part A was liquefied in a boiling water bath, and part B was added. The final pH of the medium was tested at this point and adjusted to 7.2 - 7.4 . This mixture was then dispensed in 20 ml aliquots into large sealed test tubes. Talls were stored at  $4^{\circ}C$  until needed. As each aliquot was needed, it was liquefied and cooled to  $50^{\circ}C$  in a water bath and part C was added. The total mixture was poured into a petri plate. Plates were refrigerated ( $4^{\circ}C$ ) over night before inoculation to allow maximum solidification of the medium. Correct amounts of each component used to make 1 liter of complete medium are listed in Table 2. Auxotyping media were prepared in an identical manner except that in making part B, amino acids were omitted as desired.

### Organisms

Isolates utilized in this study were obtained from the Neisseria culture bank maintained at the Montana State Health Department. Isolate #7040-74 was used as a quality control organism to judge growth on different media lots. Ten different possible mutant organisms, #'s 11066, 11348, 11205, 11226, 11341, 10643, 2763, 6124, 12461, and 10167, were used to test differentiation

TABLE 2. Quantities of components used  
to make 1 liter of complete modified medium (WSJMOM)<sup>a</sup>

Components	Quantities
Part A	
MgCl	500 ml
Agar	10 grams
Glucose	40 ml
Solution I	440 ml
Part B	
Solution III	5 ml
Amino acids and additional compounds	1 ml each
Part C	
Solution II	2 ml

<sup>a</sup>. Wong-Shockley-Johnston medium as modified by Moudy and Owsley.

capabilities of complete and incomplete media. Finally, isolate #12461 was used as a nutritional mutant to determine the amino acid necessary for its growth. Once removed from the freezer, the bacteria were maintained on chocolate agar plates and new plates were streaked every other day.

### Growth

The initial incomplete medium was prepared by omitting arginine, proline, methionine, and serine. Isolates were streaked onto plates of complete medium as well as plates lacking these four amino acids. Plates were incubated at 35.5°C in a CO<sub>2</sub> incubator, in an atmosphere containing 8-12% carbon dioxide. After 4 days of incubation, growth was observed and compared under both oblique and normal transmitted lighting.

Different incomplete media were also prepared which lacked each one of the above amino acids separately. When an isolate was found which grew on the complete and did not grow on the incomplete medium, it was then streaked onto each of these specific media which lacked only one amino acid. Growth on each plate was observed after 4 days of incubation.

## RESULTS

Attempts made in growing Neisseria gonorrhoeae on original WSJM indicated failure of the medium to sustain any growth of the control isolate. From the first set of changes made in developing an altered medium from WSJM, it was determined that glucose would be required in a 2.0% concentration (wt/vol). Concentrations of 0.75%, 1.0%, and 1.5% did not support growth as well as the medium containing 2.0% glucose. Raising the concentration of Solution II gave an increase in growth of Neisseria with each increase in concentration until 5.0% was reached. At this point, no improvement in growth was evident. A 2.0% concentration of Solution II was therefore determined to be optimal. When agar was added in a concentration of 1.0%, the medium was much too soft to be streaked with an inoculation loop. A concentration of 2.0% improved the hardening of the medium, but was still not adequate to allow streaking with ease. A 5.0% final concentration gave maximum solidification with no inhibitory effects.

In dividing stock solutions into three parts, it was necessary to keep part C (Solution II) separate, and to add it only after cooling the mixture of parts A and B to 50°C. This was necessary due to the heat sensitivity of Solution II. See Table 3 for complete final composition.

Tests conducted for detection of nutritional mutants

TABLE 3.

Composition of  
final modified medium (WSJMOM)

Compounds	Final Conc (mM)
Part A	
MgCl	8.8
Glucose	112.0
K <sub>2</sub> HPO <sub>4</sub>	23.0
KH <sub>2</sub> PO <sub>4</sub>	7.35
NaCl	85.5
Sodium acetate	25.0
Part B	
Sodium lactate	1.3
Glycerol	10.0
Oxaloacetic acid	1.52
L-Alanine	1.12
L-Arginine-hydrochloride	0.71
L-Asparagine	0.11
L-Aspartic acid	3.76
L-Cysteine-hydrochloride-water	0.35
L-Glutamic acid	8.83
L-Glutamine	.34
Glycine	.33
L-Histidine	.10
L-Isoleucine	.23
L-Leucine	.69

TABLE 3, (cont.)

Compounds	Final Conc (mM)
Part B (cont.)	
L-Lysine-hydrochloride	0.27
L-Methionine	0.10
L-Phenylalanine	0.15
L-Proline	0.43
L-Serine	0.48
L-Threonine	0.42
L-Tryptophan	0.39
L-Tyrosine	0.39
L-Valine	0.51
Hypoxanthine	0.024
Uracil	0.71
Glutathione	0.15
Biotin	0.004
Fe(NO <sub>3</sub> ) <sub>3</sub>	0.01
Part C	
Nicotinamide adenine dinucleotide	0.03
Thiamine hydrochloride	0.06
Thiamine pyrophosphate	0.04
Calcium pantothenate	0.084

resulted in the isolation of Neisseria gonorrhoeae #12461 (See Table 4). All other isolates either showed growth on both complete and incomplete media or showed no growth at all. It was determined that #12461 required arginine as part of the growth medium to sustain its growth. The isolate showed growth on the three media lacking proline, methionine, and serine, and very little if any growth on the plate lacking arginine (See Table 5, Illustrations 1 and 2).

Observing growth on plates was much easier using oblique lighting i.e. lighting at a sharp angle. Colonies are very small and lightly colored. The medium is also lightly colored and is transparent as well. Colonies were not visible or very difficult to see at best, when viewed under normal, transmitted lighting.

TABLE 4. Determination of nutritional mutants  
of Neisseria gonorrhoeae

<u>N. gonorrhoeae</u> strain	Growth on media	
	Incomplete	Complete
# 12461	-	+
11066	-	-
11348	-	-
11205	+	+
11226	+	+
11341	+	+
10643	+	+
2763	-	-
6124	+	+
10167	-	-

KEY: (+) indicates growth on the medium  
(-) indicates no growth on the medium

TABLE 5. Determination of the amino acid required for growth of N. gonorrhoeae isolate #12461 on incomplete modified (WSJMOM) medium

Amino acid missing	Growth
Proline	+
Arginine	-
Methionine	+
Serine	+

KEY: (+) indicates growth on the medium  
(-) indicates no growth on the medium

Illustration 1. Photographic illustration of growth of N. gonorrhoea isolate #12461 on plate of complete medium.



Illustration 2. Photographic illustration of growth of N. gonorrhoeae isolate #12461 on plate of media lacking arginine.



## DISCUSSION AND CONCLUSION

In our study, growth of N. gonorrhoeae medium was not abundant. At times it was so minimal that it was difficult to distinguish whether it existed at all. The incomplete medium showed very little or no growth. A problem arose in finding an isolate that could be differentiated on the two media. A group of trouble organisms was tested which was known to contain nutritional mutants. Only one organism, #12461, was distinguishable on the medium we used, which lacked arginine, proline, methionine, and serine. It was therefore not possible to do any comparative studies with different organisms since only one useable culture was found.

In comparison with the preparation of our medium, that of the WSJM is much more difficult a procedure, and is not a feasible method when quick preparation is needed in the laboratory. The final medium composition we selected as most successful supported some growth while the WSJM supported none.

Most of the success of our research lies in the development of techniques which significantly simplified preparation of the medium. The organization of components into three stock solutions made it much simpler to implement the desired variations in composition. Distribution of medium was made more efficient by dispensing it initially into aliquots of enough medium for one petri plate. In this way, variations could be prepared one plate at a time,

without necessitating the entire production of new stock solutions.

The knowledge gained through this study will provide future researchers with a useful basis for continuing development of improved media for the laboratory evaluation of problem organisms of a similar type. Results achieved show that it is possible to grow and differentiate between nutritional mutants of Neisseria gonorrhoeae. However, additional research should be done to perfect the composition and concentrations of components in order to achieve a medium which will encourage maximal growth of mutants being studied. This will be necessary before the medium can be used in quick and clear evaluation of results.

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