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Improvement in *Neisseria gonorrhoeae* culture using modified fastidious broth

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ABSTRACT

Background: Culture of *Neisseria gonorrhoeae* is essential for antimicrobial susceptibility testing and drug resistance surveillance. However, the success rate for *N. gonorrhoeae* culture from samples collected at sexually transmitted disease clinics is low. Moreover, culturing this fastidious organism can be challenging.

Objective: This study aimed to develop and evaluate a modified fastidious broth (mFB) for improved preservation and culture of *N. gonorrhoeae* that would serve as both a transport medium for specimen collection from patients with suspected gonococcal infections and a growth enhancer for *N. gonorrhoeae* culture.

Materials and methods: The mFB was evaluated using five standard bacteria strains at a concentration of 10^5, 10^3, and 10^1 CFU/ml: *N. gonorrhoeae*, *Streptococcus agalactiae*, *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus saprophyticus*. After that, the mFB was used to collect specimens from 29 patients (77 samples) at the Venereal Disease Clinic at Mae Sot Hospital in Tak Province, Thailand, between October 2023 and February 2024. A total of 77 specimens were divided into 2 groups according to the gram-negative diplococci in Gram stain: 1) positive results (8 specimens) and 2) negative results (69 specimens). Furthermore, the *N. gonorrhoeae* culture was compared by using mFB on Chocolate agar (CA) and the direct plate specimen culture on Thayer Martin agar (TMA).

Results: The results showed that the mFB could inhibit the growth of *E. coli*, *L. monocytogenes*, *S. agalactiae*, and *S. saprophyticus* but did not affect the growth of *N. gonorrhoeae* and significantly enhanced its growth. Using mFB, *N. gonorrhoeae* recovered 100% (8/8) of the positive Gram stain results from 77 clinical specimens, while direct TMA culture only recovered 75% (6/8). In addition, mFB enabled the detection of *N. gonorrhoeae* in 2 of the 69 Gram stain-negative specimens that were negative by direct TMA culture.

Conclusion: *N. gonorrhoeae* culture using mFB, followed by subculture on CA, indicated that the mFB can preserve *N. gonorrhoeae* in clinical specimens during delivery to the laboratory and promote its growth. This has the potential to improve gonorrhea diagnosis and treatment with appropriate antibiotics, as well as enhance surveillance of antimicrobial-resistant gonorrhea.

Introduction

Gonorrhea is the second most common sexually transmitted infection caused by a gram-negative bacterium called *Neisseria gonorrhoeae,* which is primarily transmitted through vaginal, oral, and anal sex. It remains a significant public health concern globally after *Chlamydia trachomatis* infection in Thailand and worldwide.[1,2] Most cases of gonorrhea are asymptomatic; an untreated infection can cause severe complications.[3] Although it is treatable and can be cured with some antibiotics, drug

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resistance in *N. gonorrhoeae* has been identified and reported.[4,5] There was evidence of antibiotic resistance in this organism due to increased treatment without carefully considering the specific antibiotics used.[4] The emergence of antimicrobial resistance in *N. gonorrhoeae* is making treatment of gonorrhea more challenging, with the risk of being untreatable. While ceftriaxone is the last option for first-line empirical monotherapy of gonorrhea, the first gonococcal isolates with ceftriaxone resistance along with high-level azithromycin resistance were identified in England and Australia in 2018.[6] Furthermore, the increasing minimal inhibitory concentration (MIC) of ceftriaxone, cefixime, and azithromycin for *N. gonorrhoeae* antimicrobial susceptibility was reported from Thailand in 2023.[7] The rational use of antibiotics and the development of new ones are critical to reducing this imminent threat.

N. gonorrhoeae culture is essential for antimicrobial susceptibility testing in infection treatment. It provides essential information for appropriate gonorrhea treatment and is vital for monitoring antimicrobial resistance. Specimen collection for *N. gonorrhoeae* culture must be done by a clinician who uses a swab or loop to collect specimens from patients before being sent to the laboratory for gonorrhea diagnosis using Gram stain and culture with special media. Moreover, *N. gonorrhoeae* is a fastidious pathogen and does not tolerate dehydration, which does not grow well in standard liquid media.[8] Thayer-Martin agar (TMA) is a unique culture medium for *N. gonorrhoeae,* providing the necessary nutrients to enhance *N. gonorrhoeae* growth. Therefore, the utilization of an enrichment broth medium is recommended to improve the sensitivity of cultures that may contain small numbers of these organisms.[9]

To address this problem, we developed a new liquid enrichment medium: modified fastidious broth (mFB), formulated to preserve and enhance the growth of *N. gonorrhoeae* from clinical specimens during transportation to the laboratory and to select and enhance *N. gonorrhoeae* growth in culture.

Materials and methods

Bacterial strains

Five standard bacteria strains: *N. gonorrhoeae* ATCC 49226, *Escherichia coli* ATCC 25922, *Staphylococcus saprophyticus* ATCC 19701, *Listeria monocytogenes* ATCC 19115, and *Staphylococcus agalactiae* ATCC 12386 were included in this study. They were obtained from the Thailand Biodiversity Center and the National Center for Genetic Engineering and Biotechnology. Freshly collected cultures were obtained from the original frozen stock cultures on chocolate agar (CA) for 24 hours to ensure the purity of colonies. They used VITEK® 2 COMPACT (Biomerieux, France) for identification before testing.

Clinical strains

The clinical specimens were collected from 29 patients (5 males and 24 females) with urogenital symptoms, primarily urethral or vaginal discharge, who visited the Venereal Disease Clinic at Mae Sot Hospital in Tak Province, north-west Thailand, between October 2023 and February 2024. The Naresuan University Institutional Review Board ethically approved this study, and informed consent was obtained from all patients before specimen collection. Seven patients (5 males and 2 females) were diagnosed with gonorrhea after finding gram-negative dipplococci by Gram stain. They were treated with 500 mg of ceftriaxone by injection. Twenty-two patients (females) were diagnosed with non-gonorrhea infections and received treatment based on their symptoms.

A total of 77 specimens were collected from 29 patients, composed of the urethra (29), cervical (24), and vagina (24), as shown in Table 1. All specimens were screened for gonorrhea infection before culture using a Gram stain. Then, they were inoculated on Thayer-Martin agar and in modified fastidious broth for *N. gonorrhoeae* culture.

Table 1. Type of specimens and Gram stain results of 77 specimens**.**

Sex (N)	Gram-negative diplococci						Total
	Positive (N=8)			Negative (N=69)			
	Urethra	Cervix	Vagina	Urethra	Cervix	Vagina	
Male (5)	5	0	0	0	0	0	5
Female (24)	1	2	0	23	22	24	72
Total (29)	6	2	0	23	22	24	77

Modified fastidious broth

The fastidious broth to enhance and amplify fastidious bacteria, especially *Neisseria* spp., was developed using Brain Heart Infusion broth as a liquid medium base, supplemented with hematin with an X factor (hemin). This solution was autoclaved at 121 °C for 15 min. Then, the Isovitalex Enrichment, containing vitamins, amino acids, co-enzymes, dextrose, ferric ions, and other elements, was added. Moreover, the Vancomycin-Colistin-Nystatin (V-C-N) inhibitor was used to suppress normal flora including fungi. After fastidious broth preparation, 5 mL aliquots were dispensed into 10 ml sterile culture tubes and stored at 4 °C.

Evaluation of modified fastidious broth in selection and growth enhancment

Five bacterial suspensions (*N. gonorrhoeae* ATCC 49226, *E. coli* ATCC 25922, *S. saprophyticus* ATCC 19701, *L. monocytogenes* ATCC 19115, and *S. agalactiae* ATCC 12386) were adjusted to the 0.5 McFarland standard

using sterile saline (0.45% NaCl). Ten-fold serial dilutions of 10^5, 10^3, and 10^1 CFU/mL were prepared in triplicate. The bacterial growth-enhancing ability of mFB was determined by visual turbidity after 2-, 24-, and 48-hr incubation at 35 °C with 5% CO_2, compared to mFB that did not contain organisms. Moreover, the typical colonies of each organism after subculture on CA were observed and identified using Gram stain and VITEK® 2 COMPACT (Biomerieux, France) after incubation at 35 °C with 5% CO_2 for 24 and 48 hrs.

Comparison of N. gonorrhoeae culture from clinical specimens between direct culture and modified culture using mFB

Direct culture on Thayer Martin agar

A total of 77 specimens were directly plated on TMA within 1 hour after collection and incubated at 35 °C with 5% CO_2 according to the Manual of Clinical Microbiology, 7th Edition.[10] The typical colonies of *N. gonorrhoeae* on TMA were observed at 24 and 48 hrs, identified using Gram stain and oxidase testing, and confirmed using NH VITEK® 2 COMPACT (Biomerieux, France).

Modified culture using mFB

In the same way, 77 specimens were collected and immediately put into mFB. Then they were incubated at 35 °C with 5% CO_2 within 1 hr after collection. The signs of growth were monitored by observing turbidity and subculturing on CA after 2-, 24-, and 48-hr incubation at 35 °C with 5% CO_2. The typical colonies of *N. gonorrhoeae* on CA were observed at 24 and 48 hrs and were identified in the same manner as the previously identified colonies on TMA.

Results

Preservation, selection, and enhancing growth of mFB

The ability of mFB to enhance the growth is shown in Table 2. It indicates that mFB effectively selected, supported, and enhanced the growth of *N. gonorrhoeae*. All inoculums of *N. gonorrhoeae* were visually detectable by turbidity within 24 hrs of incubation. In contrast, none of the inoculums of the four standard bacterial strains (*E. coli*, *S. saprophyticus*, *L. monocytogenes*, and *S. agalactiae*) were detectable. This mFB also enhanced the growth of *N. gonorrhoeae* better than four bacterial strains after subculture on CA. mFB completely inhibited the growth of gram-negative bacteria such as *E. coli*, which could not grow in the mFB. In addition, the mFB could support and enhance *N. gonorrhoeae* growth in the early incubation (2 hrs), with the colonies present on CA at approximately 10^3 and 10^5 CFU/ml. In contrast, there was a bit of enhanced growth in the early incubation (2 hrs) of *L. monocytogenes* and *S. agalactiae* at approximately 10^5 CFU/mL concentration, and their growth rates began to decline after 24 hrs of incubation.

Table 2. Growth enhancing of modified fastidious broth.

Organisms	Inoculum (CFU/ml)	Turbidity			Growth results on CA		
		2 hrs	24 hrs	48 hrs	2 hrs	24 hrs	48 hrs
E. coli	10^5	×	×	×	-	-	-
	10^3	×	×	×	-	-	-
	10^1	×	×	×	-	-	-
S. saprophyticus	10^5	×	×	×	+ $(1)^3$	-	-
	10^3	×	×	×	-	-	-
	10^1	×	×	×	-	-	-
L. monocytogenes	10^5	×	×	×	+ $(1)^4$	+ $(1)^2$	+ $(1)^1$
	10^3	×	×	×	+ $(1)^2$	+ $(1)^1$	-
	10^1	×	×	×	-	-	-
S. agalactiae	10^5	×	×	×	+ $(1)^4$	+ $(1)^2$	-
	10^3	×	×	×	+ $(1)^1$	-	-
	10^1	×	×	×	-	-	-
N. gonorrhoeae	10^5	×	✓	✓	+ $(1)^3$	+ $(1)^4$	+ $(1)^4$
	10^3	×	✓	✓	+ $(1)^2$	+ $(1)^4$	+ $(1)^4$
	10^1	×	✓	✓	-	+ $(1)^4$	+ $(1)^4$

***Note**: (✓): visually (turbidity) detecting, (×): no visually (turbidity) detecting, (+): organism growth on CA after subculture from incubated mFB, (-): organism no growth on CA after subculture from incubated mFB, (A)[b], A: numbers in parentheses represent the earliest times (in days) at which growth, b: organism grading on CA (1; rare (<10 colonies), 2: few (organism growth 1/4 plate, >10 colonies), 3: moderate (organism growth 2/4 plate), 4: many [organism growth 3/4 plate]).*

Comparison of N. gonorrhoeae culture from clinical specimens between TMA and mFB

The *N. gonorrhoeae* colonies on CA were observed after subculture from mFB at a 2-hr incubation period in all 8 specimens (100%; 6 specimens from the urinary tract and 2 specimens from the cervix), as shown in Table 3. In contrast, when using directed culture on TMA, the *N. gonorrhoeae* colonies were found in 6 out of 8 specimens (75%; 4 specimens from the urinary tract and 2 specimens from the cervix), while 2 out of 8 specimens from the male urethra (25%) showed no signs of *N. gonorrhoeae* growth.

For 69 samples that did not find gram-negative diplococci (gram stain negative results), there were no growth of *N. gonorrhoeae* when using directly cultured TMA. However, *N. gonorrhoeae* growth was observed on CA after subculture from two of the mFB incubated specimens from the vaginal specimens (2.90%). When comparing the culture results using the Z test, there was a non-significant difference between the two methods (Z-score of 1.0564 and a p=0.289) (data not shown).

All 6 *N. gonorrhoeae* isolates growth on TMA and 10 *N. gonorrhoeae* isolates growth on CA (after subculture using mFB) were confirmed by Gram stain and identification using oxidase test, catalase test, and VITEK 2 NH Identify CARD (VITEK® 2 COMPACT, Biomerieux). Moreover, all ten isolates were tested for antimicrobial susceptibility by disk diffusion with ciprofloxacin (5 µg), tetracycline (30 µg), ceftriaxone (30 µg), and penicillin (10 units).[11] They showed that all isolates were resistant to ciprofloxacin, tetracycline, and penicillin but still susceptible to ceftriaxone (data not shown).

Table 3. Comparison of *N. gonorrhoeae* culture between using TMA and mFB.

Type of culture media	Gram's stain positive (N=8)		Gram's stain negative (N=69)		Total
	Culture Positive	Culture Negative	Culture Positive	Culture Negative	
Modified Fastidious broth (followed by subculture on CA)					
Urethra	6	0	0	23	29
Cervix	2	0	0	22	24
Vagina	0	0	2	22	24
Total	**8 (100%)**	**0**	**2 (2.90%)**	**67 (97.10%)**	**77**
Thayer Martin agar					
Urethra	4	2	0	23	29
Cervix	2	0	0	22	24
Vagina	0	0	0	24	24
Total	**6 (75%)**	**2 (25%)**	**0**	**69 (100%)**	**77**

***Note**: Gram stain positive: found gram-negative diplococci, Gram stain negative: not found gram-negative diplococci, Culture Positive: N. gonorrhoeae growth on culture media, Culture negative: no N. gonorrhoeae colonies on culture media.*

Discussion

The enhancing ability of mFB was examined for visual signs of growth both in the mFB tube and after subculture on CA. We found that the mFB preserved and enhanced recovery of a small number of *N. gonorrhoeae* is significantly intriguing (Tables 2 and 3). The results demonstrated that mFB, with a low inoculum of 10^1 CFU/ml, improved the recovery of *N. gonorrhoeae*, allowing them to grow in large numbers on CA after subculturing from incubated mFB. On the other hand, a report by Cartwright CP et al. found that FB enhanced recovery of 10^2 CFU/ml by showing visual signs of growth in FB after a 96-hour incubation period.[12] Moreover, the data demonstrated that VCN inhibitors in the mFB can affect the growth of other organisms, including *E. coli*, *S. saprophyticus*, *L. monocytogenes*, and *S. agalactiae*, which are usually found in vagina and urinary tract systems.[13-15] However, these inhibitors did not affect *N. gonorrhoeae* growth.[16] This may be due to *N. gonorrhoeae* having intrinsic resistance mechanisms to vancomycin, trimethoprim, and colistin.[17] This implied the possibility of using mFB as a transport and selective medium for *N. gonorrhoeae*.

For *N. gonorrhoeae* culture from clinical specimens, the efficiency of mFB in growth enhancement was more significant than using a direct plate specimen on TMA (Table 3). However, statistical analysis indicated no significant difference between the two media. One limitation of this study is the small sample size. Future studies should aim to collect more samples, particularly from patients with *N. gonorrhoeae* infections.

In addition, the colonies of *N. gonorrhoeae* were detected on CA after subculturing from the mFB of two specimens with negative gram stain results initially. Possible causes could be an insufficient specimen, a small organism, or smear preparation.[18] These suggest that its ability was both to preserve and enhance the growth of this organism. The incubation in mFB before subculture on CA could improve the likelihood of *N. gonorrhoeae* detection in the specimen compared to direct culture on TMA. This research demonstrated that the mFB enhanced the growth of small organism specimens and enabled their cultivation on readily available CA medium, potentially eliminating the need for TMA in culture procedures. Furthermore, a microbiology laboratory can prepare this mFB in-house

from the essential media or supplement available, which can help reduce the cost of *N. gonorrhoeae* culture.

This study has attempted to develop a liquid culture medium known as modified fastidious broth (mFB). The findings suggest that mFB can preserve the *N. gonorrhoeae* in specimens during transport to the laboratory. Additionally, this mFB could enhance the likelihood of detecting *N. gonorrhoeae* in culture from the patient's specimen. The inhibitors in mFB are likely to inhibit the growth of any bacteria that may be contaminants. However, it did not affect the growth of *N. gonorrhoeae*. As a result, we can test for drug susceptibility, which will provide future guidelines for treatment and surveillance of antibiotic resistance.

Conclusion

In summary, culturing *N. gonorrhea* using mFB followed by subculture on CA may improve gonorrhea diagnosis. It also provides the low cost of medium preparation, simplicity in specimen collection, and the ability to conduct drug susceptibility testing. As a result, it not only facilitates appropriate gonorrhea treatment but also supports surveillance of antimicrobial resistance in *N. gonorrhea*.

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