Evaluation of GenoFlow Bacterial Meningitis Array Testing Kits with Reference to Conventional Method for the Diagnosis of Bacterial Meningitis Pathogens

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ABSTRACT

Bacterial meningitis continuously threatens public health with its high morbidity and mortality rates. Due to complex etiological and aggressive nature of bacterial meningitis, a rapid and sensitive diagnostic method is needed before deciding a proper treatment, e.g., the use of antibiotics. In this study, we collected 604 clinical samples from Hungary and Shenzhen, China and compared the performance of DiagCor's GenoFlow Bacterial meningitis array test kit against the gold standard, i.e., bacterial culture and latex agglutination. The results showed that the array test kit had a 96.5% concordant rate with the reference methods. We further analyzed the discrepant results using PCR and Sanger sequencing and confirmed that the array test kit indeed showed 100% sensitivity and 100% specificity for all the pathogens detected by various reference methods. In conclusion, the GenoFlow Bacterial meningitis array test kit represented a sensitive, accurate and rapid laboratory method to help of diagnosis suspected bacterial meningitis cases. The use of this multiplex detection array test could provide prompt and appropriate antibiotic treatment in managing meningitis patients.

Keywords: *Bacterial Meningitis, Cerebrospinal Fluid, Molecular Diagnostics, Multiplex PCR, Flow Through Hybridization*

Abbreviations

BLAST, Basic Local Alignment Search Tool; CSF, cerebrospinal fluid; EFM, *Enterococcus faecium*; EFS, *Enterococcus faecalis*, EC, *Escherichia coli* K1; WBC, white blood cell; PCR, polymerase chain reaction; HA, *Haemophilus influenza*, NM, *Neisseria meningitidis*, SP, *Streptococcus pneumonia*; SAG, *Streptococcus agalactiae*; SPY, *Streptococcus pyogenes*; PA, *Pseudomonas aeruginosa*; LM, *Listeria monocytogenes*; MP, *Mycoplasma pneumoniae*; SE, *Staphylococcus epidermidis* and Limit of detection (LOD);

INTRODUCTION

Bacterial meningitis causes significant morbidity and mortality, and it continually threatens public health globally. The mortality rate of bacterial meningitis can be as high as 30%, especially in newborns and older patients,[1] and it is associated with various bacterial pathogens. For example, *Haemophilus*: *influenzae* is one of the major causes of bacterial meningitis, and this pathogen has led to 45% of all bacterial meningitis cases in the US before the launch of the conjugated vaccine.[2] *Streptococcus pneumoniae* is another important bacterium that causes meningitis, and it accounted for 60% of the total meningitis cases in US and Turkey.[1, 3] It was also the most observed meningitis pathogen in Italy, Israel and Brazil.[4-6] *Neisseria meningiditis* infection rate could be up to 1% of the population, and severe infection rate was found globally including Brazil, Nepal, China, and several sub-Saharan African nations.[7-11] Due to its high infection rate, an 'African Meningitis belt' was mapped out.[12] *N. meningitidis* associated meningitis cases were also common in some developed countries such as the United States.[13] *Streptococcus agalactiae* (GBS), on the other hand, is an important pathogen which caused 59% of neonatal meningitis cases in France,[14] and it was the most common cause of neonatal meningitis in England.[15]

Owing to the complexity and high mortality rate of bacterial meningitis infection, a rapid diagnostic method is crucial to provide proper treatment. Currently, the most common bacterial meningitis diagnosis is based on white blood cell (WBC) counting of CSF (cerebrospinal fluid), turbidity observation, Gram staining, latex agglutination,

and bacterial culture.[16] These conventional diagnostic methods are relatively slow with low sensitivity, which may not be able to cope with the aggressive and complex nature of bacterial meningitis infection. On the other hand, the nucleic acid amplification test represents a quick and versatile diagnostic tool against bacterial meningitis (PCR) over the bacterial culture method as a diagnostic tool has been demonstrated previously.[16]

GenoFlow Bacterial meningitis array test kit is a PCR based diagnostic test kit, which was developed specifically to detect the most common pathogens found in bacterial meningitis infection by DiagCor **(Table 1)**. Amplified bacterial DNA in the CSF sample is hybridized with specific probes on the nitrocellulose membrane by flow-through hybridization technique. This technology enables the simultaneous detection of multiple targets in the sample and shortens the hybridization time to 30 minutes dramatically.[17] The potential uses of this test kit include rapid laboratory diagnosis of suspected bacterial meningitis cases, revealing various infection cases, and providing clues for proper antibiotic treatment.

Table 1. Twelve (12) Most Common Bacteria Causing Human Meningitis that Included in the GenoFlow Bacterial Meningitis Array Testing Kit

Target pathogens	Abbreviations
Haemophilus influenzae	HI
Neisseria meningitidis	NM
Streptococcus pneumoniae	SP
Streptococcus agalactiae	SAG
Streptococcus pyogenes	SPY
Pseudomonas aeruginosa	PA
Listeria monocytogenes	LM
Mycoplasma pneumoniae	MP
Enterococcus faecium	EFM
Enterococcus faecalis	EFS
Staphylococcus epidermidis	SE
Escherichia coli K1	EC

This study aimed to evaluate the performance of the DiagCor's GenoFlow Bacterial meningitis array test kit in comparison with different traditional diagnostic methods such as bacterial culture and latex agglutination tests.

MATERIALS AND METHODS

Clinical Specimens

Six hundred and four (604) CSF samples in total were collected from patients with suspected bacterial meningitis. Among them, 98 samples were collected from St. Laszlo Hospital in Hungary and 506 samples from Shenzhen Children's Hospital in China. The samples reached the laboratory in the respective hospital within four hours after lumbar puncture and underwent conventional microbiologic testing, including Gram staining, WBC counting, and bacterial culture. All visibly cloudy samples were tested by latex agglutination. Aliquot of two hundred microliters (200 µL) of CSF samples were stored at -80°C for a comparison study using DiagCor's GenoFlow Bacterial meningitis array test kit. Consent was obtained from all patients or their parents for those younger than the age of 18 years old.

Synthetic Plasmid Generation and Bacterial Genomic DNA

Synthetic plasmids containing sequences specific to the targets were constructed using pUC57 vector. The plasmids were manufactured from GenScript (Piscataway, USA). Bacterial genomic DNA was purchased from ATCC (Manassas, USA). *Haemophilus influenza* (ATCC® Number: 51907D); *Neisseria meningitides* (ATCC® Number: 53415D-5); *Streptococcus pneumonia* (ATCC® Number: BAA-255D-5); *Streptococcus agalactiae* (ATCC® Number: BAA-611D-5); *Streptococcus pyogenes* (ATCC® Number: BAA-1315D-5); *Pseudomonas aeruginosa* (ATCC® Number: 47085D-5); *Listeria monocytogenes* (ATCC® Number: BAA-679D-5); Mycoplasma *pneumoniae* (ATCC® Number: 29342D); *Enterococcus faecium* (ATCC® Number: 51559D-5); *Enterococcus faecalis* (ATCC® Number: 700802D-5); *Staphylococcus epidermidis* (ATCC® Number: 35984D-5).

Bacterial Culture and Latex Agglutination

In Hungary, the samples from St. Laszlo Hospital were cultured on Columbia blood agar and chocolate agar and parallel in brain heart infusion broth (Bio-Merieux, Marcyl' Etoile, France). Incubation time was 72 hours. Identification was with MALDI-TOF (Bio-Merieux, Marcyl' Etoile, France). Susceptibility is according to EUCAST recommendations. The samples collected from Shenzhen Children's Hospital in China were cultured on blood agar plates and chocolate blood plate for 72 hours at 35°C in a 5-10% CO_2 atmosphere. The observed colonies were identified using the Vitek2 Compact system (Bio-Merieux, Marcyl' Etoile, France) according to the manufacturer's instruction. For latex agglutination test performed in Hungary, the samples were evaluated by using Wellcogen Bacterial Antigen Rapid Latex Agglutination Test (ThermoFisher Scientific, MA, USA) according to the manufacturer's instruction.

DNA Extraction, Amplification and Detection Using DiagCor's GenoFlow Bacterial Meningitis Array Testing Kit

DNA was isolated from 200 µL CSF samples using the QIAamp DNA Blood Mini Kit (No. 51104; Qiagen, Hilden, Germany) according to the manufacturer's instruction. The extracted DNA was further analyzed with the GenoFlow Bacterial meningitis array test kit to detect the presence of pathogens in the clinical samples according

to the manufacturer's manual. Briefly, a 25 µL reaction containing 5 µL plasmid DNA, 14.1 µL PCR reaction mix, 2.5 µL primer mix, 1 µL GC enhancer, 2 µL internal amplification control and 0.4 µL DNA Taq polymerase was set up for each sample. The PCR mixtures were amplified under the following thermal cycling profile: initial denaturation at 95°C for 5 mins; 45 cycles at 95°C for 30s, annealing at 58°C for 30s, extension at 72°C for 30s; and a final extension step at 72°C for 10 min. Following PCR amplification, the amplicons were subjected to flow-through hybridization as instructed. Bacterial genomic DNA (10^6 copies) and human DNA (20 ng, equivalent to 5.79×10^3 copies) were used to determine the cross-reactivity of the detection signals. The obtained results were used to compare with those from culture and latex agglutination results.

Sanger Sequencing

Samples with discrepant results were further analyzed by standard Sanger sequencing using a pair of 16S rRNA primers or UMD-Universal (No. U-010-048, Molyzm, Bremen, Germany) according to our in-house method or the manufacturer's instruction, respectively. Amplicons were purified using PureLink QuickGel Extraction and PCR purification combo kit (No. K220001, Thermo Fisher Scientific, Waltham, MA, USA) and were sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 DNA analyzer (PE Applied Biosystems). Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Analytical Sensitivity and Specificity of DiagCor's GenoFlow Bacterial Meningitis Array Testing Kit

The upper and lower LOD for all 12 target pathogens **(Table 1)** of the GenoFlow Bacterial meningitis array test kit was determined. Serially diluted synthetic plasmids were used to determine the analytical sensitivity and specificity. The detection range of the GenoFlow Bacterial meningitis array test kit was from 100 to 10^6copies, except PA that can only achieve 500 copies at the lowest LOD. Genomic DNA of human and other reference bacteria were used to test the specificity of the array test, and not cross-reactivity signal was observed (data not shown).

Comparison of GenoFlow Bacterial Meningitis Array Testing kit Verse Conventional Culture and CSF Latex Agglutination Results

A total of 604 samples were collected from patients with suspected bacterial meningitis, 44 positive and 560 negative samples were identified using reference methods, including traditional culture and latex agglutination as shown in **Table 2**. Of the 44 positive samples, 31 samples were determined as positive by both reference method and GenoFlow Bacterial meningitis array test kit. Twelve of the leftover 13 samples were identified as negative results since the assay panel did not include the causative pathogens. Reference methods detected the remaining positive sample as *Staphylococcus aureus*, while GenoFlow Bacterial meningitis array test identified them as *Streptococcus pyogenes*. The Sanger sequencing confirmed the results of GenoFlow kit. The sequencing result was concordant with the array test with 98-100% matched as SPY in the BLAST search (data not shown). Of the 560 negative samples, 19 discordant results were detected in the presence of bacterial DNA by the GenoFlow Bacterial meningitis array test. These discrepant samples were further analyzed by Sanger sequencing, and the identities of all these bacteria were concordant with the GenoFlow Bacterial meningitis array test results.

Table 2. Comparison Results between Reference Methods (bacterial culture and latex agglutination) and GenoFlow Bacterial Meningitis Array Testing Kit

Pathogens	No. of positive by reference methods	No. of positive by GenoFlow Bacterial meningitis array test kit	** No. of Discrepant result
Haemophilus influenzae	0	2	2
Neisseria meningitidis	8	9	1
Streptococcus pneumoniae	16	17	1
Streptococcus agalactiae	1	4	3
Streptococcus pyogenes	0	1	1
Pseudomonas aeruginosa	0	3	3
Listeria monocytogenes	0	2	2
Staphylococcus epidermidis	6	11	5
Escherichia coli K1	0	2	2
*Other pathogens	13	0	n/a
Total no. of positives	44	51	n/a
Total no. of negatives	560	553	n/a
Total no. of samples	604	604	20

* Other pathogens were out-panel pathogens in GenoFlow Bacterial meningitis array test kit which includes 1 *Streptococcus equinus*, 1 *Staphylococcus hominis*, 4 *Staphylococcus aureus*, 2 *Coagulase-negative Staphylococcus*, 1 *Staphylococcus haemolyticus*, 1 *Bacteroides thetaiothaomicron*, 1 Cryptococcus neoformans, 1 Ochrobactrum anthropic, and 1 *Acinetobacter lwoffii*.

** All the discrepant samples were subjected to Sanger sequencing, and results were concordant with GenoFlow Bacterial meningitis array test kit.

Clinical Sensitivity and Specificity of DiagCor's GenoFlow Bacterial Meningitis Array Testing Kit

Clinical sensitivity and specificity of GenoFlow Bacterial meningitis array test kit were calculated according to the results obtained from reference methods as shown in **Table 3**. The overall sensitivity and specificity were 100%. The sensitivity and specificity of individual targets were also determined and listed in **Table 4**. However, the sensitivity and specificity of targets for MP, EFM, and EFS could not be evaluated due to the lack of clinical samples.

Table 3. Clinical Performance of GenoFlow Bacterial Meningitis Array Testing Kit				
		Reference methods (Bacterial culture and latex agglutination) and Sanger sequencing		
GenoFlow Bacterial meningitis Array test kit	N = 604	Positive	Negative	
	Positive	51	0	Positive Predictive Value = 100%
	Negative	0	553	Negative Predictive Value = 100%
		Sensitivity = 100%	Specificity = 100%	
		False Negative Rate = 0%	False Positive Rate = 0%	

Clinical performance of GenoFlow Bacterial meningitis array test kit was evaluated by reference methods, including bacterial culture, latex agglutination as the gold standard. The discrepant result was further analyzed using Sanger sequencing.

Table 4. Sensitivity and Specificity of Individual Target in GenoFlow Bacterial Meningitis Array Testing Kit		
Targets	**Sensitivity (%)**	**Specificity (%)**
Haemophilus influenzae	100	100
Neisseria meningitidis	100	100
Streptococcus pneumoniae	100	100
Streptococcus agalactiae	100	100
Streptococcus pyogenes	100	100
Pseudomonas aeruginosa	100	100
Listeria monocytogenes	100	100
Staphylococcus epidermidis	100	100
Escherichia coli K1	100	100

Sensitivity and specificity were calculated from both reference and Sanger sequencing results. The sensitivity and specificity of MP, EFM, and EFS cannot be determined in this study.

Incidence Rate of Pathogens Detected by DiagCor's GenoFlow Bacterial Meningitis Array Testing Kit in Hungary and Shenzhen

We expect the prevalence of bacterial meningitis varied in different countries and age groups. In this study, the most common pathogens found in the United St. Isvan and Laszlo Hospital in Hungary were SP and NM, which are found frequently in bacterial meningitis cases worldwide **(Figure 1A)**. The age of these patients in the hospital in Hungary varied from newborn to 93 years old. While in the Shenzhen region of China, SE was found to be the most prevalent bacterial causing pathogen, followed by SP and SAG **(Figure 1B)**.

DISCUSSION

Patients with bacterial meningitis require proper and prompt treatment to avoid adverse clinical outcomes, such as brain damage, hearing loss, or learning disabilities.[18] Therefore, it is essential to identify the causative agent(s) to initiate appropriate antibiotic treatment. Routine laboratory practice employs conventional cytological examinations, e.g., Gram staining, cell counting, and latex agglutination tests for immunological identification, to diagnose bacterial meningitis in suspected cases. Although these methods are relatively rapid and low-cost, they often lack specificity, and their sensitivities might diminish with preceding empirical antibiotic therapy.[19] CSF culture is considered as the gold standard to identify causative pathogens in bacterial meningitis. However, the long reporting time of CSF culture prevents specific antibiotic treatment in early disease stage. Molecular diagnostic assays were recently applied in clinical decision making and surveillance.[20, 21] Several detection kits are currently available commercially based on multiplex PCR principle, while the GenoFlow Bacterial meningitis array test employs flow-through hybridization technology and offers the capability to detect 12 most prevalence bacteria causing meningitis simultaneously. Furthermore, we found that the LOD revealed the detection range to as low as 100 copies of plasmid DNA and 500 copies for PA, which is comparable to other attainable multiplex array test series developed to identify common viral and bacterial

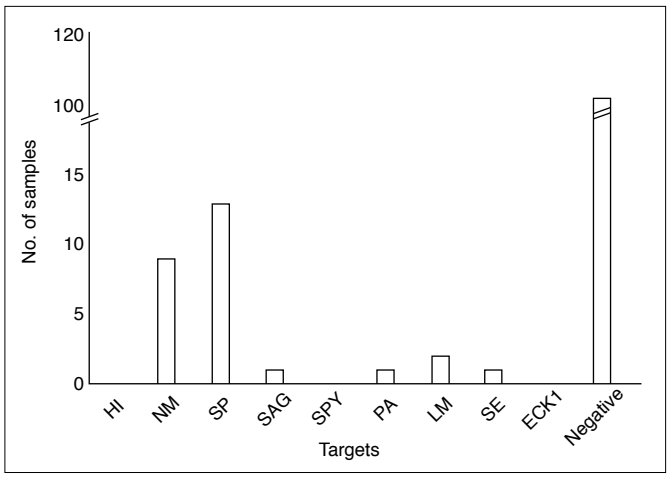

Figure 1A. *Distribution of sample identity detected in GenoFlow bacterial meningitis array test kit collected in St. Laszlo Hospital, Hungary*

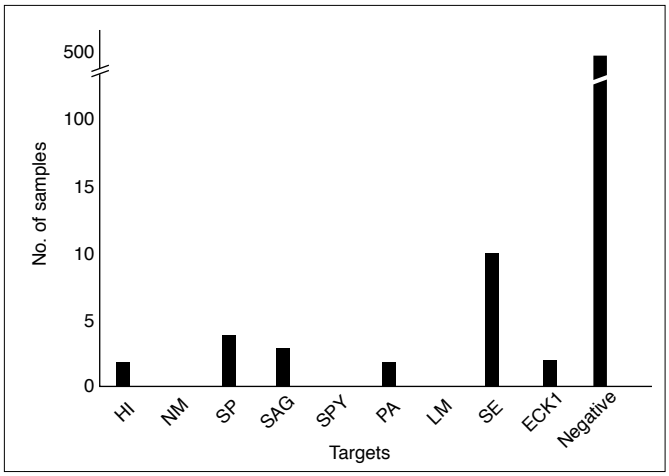

Figure 1B. *Distribution of sample identity detected in GenoFlow bacterial meningitis array test kit collected in Shenzhen Children's Hospital, China*

pathogens. For instance, the GenoFlow HPV Array Test Kit and the Genoflow DR-MTB Array Test Kit, are meanwhile being used and evaluated in different clinical laboratories.[22-24]

In this study, it showed that the GenoFlow Bacterial meningitis array test offered high detection sensitivity and specificity for nine pathogens observed in the clinical samples suspected with bacterial meningitis **(Table 3)**. Excluding pathogens that were out of the detection panel, the overall concordant rate was 3.5% in comparison to the reference methods. Furthermore, 19 out of 20 discrepant samples were identified as negative in reference methods but positive in the GenoFlow Bacterial meningitis array test. All the 19 discordant samples were further confirmed as positive with sequencing data. The discrepancies observed might be due to the low amount of pathogens presented in CSF. GenoFlow Bacterial meningitis array test was sensitive enough to detect a trace amount of pathogens presented in the samples. It is critical to provide a prompt and precise antibiotic treatment to minimize treatment failure rate and reduce clinical complications. The workflow of the array test can be completed within three hours, whereas the golden standard culture method takes up to few days for pathogen identification. This test kit provides a significant advantage for pathogen detection as it is highly sensitive, accurate and fast. Although this assay offers the capability to identify 12 specific pathogens, it is still restricted to a discrete number of targets based on the most common meningitis-causing pathogens. In this study, the array test kit was unable to identify any pathogen in 14 positive clinical samples, which were later confirmed as the pathogens not included in the array. It showed the limitation of this array test kit to detect rare bacteria strain out of the panel. We recognize that molecular assay could be used as an adjunct to culture method, which will remain essential for antibiotic susceptibility testing.

The epidemiology of bacterial meningitis has changed substantially since the administration of conjugate vaccines.[1, 25, 26] The statistical data from this study suggested that, in children, SP and NM remained as the leading cause of bacterial meningitis in Hungary while SE was the most common pathogens in Shenzhen region in China. Previous studies demonstrated that SP, NM, and HI are the most prevalence etiological agents of bacterial meningitis.[27] Vaccination strategy targeting the most common community-acquired pathogen was introduced in developed countries, which were associated with the prominent reduction of bacterial meningitis cases for disease control.[28] On the other hand, bacterial meningitis is mostly found for the child, elderly and immunocompromised patients.[27] The epidemiology of bacterial meningitis has to be studied thoroughly in different regions as well as various age groups to develop a proper vaccination strategy which can effectively control the most common community-acquired pathogens that cause bacterial meningitis.

Viral meningitis accounts for most cases of acute meningitis. Bacterial and viral meningitis cannot be reliably differentiated clinically, and all suspected cases should be referred to a hospital.[29] In most cases, no treatment is necessary for viral meningitis. Certain medications can be useful, depending on the virus that caused the infection. Therefore, there are considerable benefits in making the differentiation between bacterial and viral meningitis swiftly, in terms of both reducing antibiotic usage and hospital admission. A more comprehensive molecular panel is needed to provide immediate and precise therapy to the patients.

In conclusion, our results demonstrated that the GenoFlow Bacterial meningitis array test kit had high sensitivity and specificity for the 12 most common bacteria which cause bacterial meningitis. It can be used to provide rapid identification of pathogens in patients with suspected bacterial meningitis to initiate suitable antibiotic treatments promptly and offer better patient management.

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Declaration

The author declares that the research methods and results obtained in this study are pertinent and do not deliberately favor any brand mentioned in the manuscript.

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