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Rapid diagnosis of bacterial meningitis by nanopore 16S amplicon sequencing: A pilot study

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ABSTRACT

Early administration of antibiotics is crucial in the management of bacterial meningitis. Rapid pathogen identification helps to make a definite diagnosis of bacterial meningitis and enables tailored antibiotic treatment. We investigated if the 16S amplicon sequencing performed by MinION, a nanopore sequencer, was capable of rapid pathogen identification in bacterial meningitis. Six retrospective cases of confirmed bacterial meningitis and two prospective cases were included. The initial cerebrospinal fluid (CSF) samples of these patients were used for the experiments. DNA was extracted from the CSF, and PCR was performed on the 16S ribosomal DNA (16S rDNA). Sequencing libraries were prepared using the PCR products, and MinION sequencing was performed for up to 3 h. The reads were aligned to the bacterial database, and the results were compared to the conventional culture studies. Pathogenic bacteria were successfully detected from the CSF by 16S sequencing in all retrospective cases. 16S amplicon sequencing was more sensitive than conventional diagnostic tests and worked properly even in antibiotics-treated samples. MinION sequencing significantly reduced the turnaround time, and even 10 min of sequencing was sufficient for pathogen detection in certain cases. Protocol adjustment could further increase the sensitivity and reduce the turnaround time for MinION sequencing. Finally, the prospective application of MinION 16S sequencing was successful. Nanopore 16S amplicon sequencing is capable of rapid bacterial identification from the CSF of the bacterial meningitis patients. It may have many advantages over conventional diagnostic tests and should therefore be applied in a larger number of patients in the future.

1. Introduction

Acute bacterial meningitis is a life-threatening infectious disease, and patients with this disease require immediate medical assessment and treatment. Nevertheless, the disease continues to occur worldwide, including in high-income countries, causing substantial morbidity and mortality.(Brouwer et al., 2012; van de Beek et al., 2012) There are limited options for the treatment of bacterial meningitis, and the early administration of antibiotics is most important to increase survival and reduce morbidity.(Proulx et al., 2005; van de Beek et al., 2012) Corticosteroids might offer a small benefit for reducing mortality and morbidity, but potential adjunctive therapies, such as glycerol and hypothermia, have not been demonstrated to be beneficial in randomized controlled trials.(McGill et al., 2016) Therefore, making a rapid diagnosis is the crucial step in the management of bacterial meningitis.

Pathogen identification by deep sequencing is increasingly applied to microbial research and clinical diagnostics. Metagenomic sequencing enables unbiased, hypothesis-free, and rapid detection of all types of microbes, including rare or novel pathogens that were not isolated by conventional diagnostic tests.(Hoffmann et al., 2015; Kosoy et al., 2015; Naccache et al., 2015; Wilson et al., 2014, 2018) However in particular clinical samples, low abundance of the microbial sequences troubles identifying the pathogen by shotgun metagenomic sequencing. Meanwhile, amplicon-based metagenomic sequencing could be a better

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approach in certain circumstances. Especially, 16 s ribosomal DNA (16S rDNA) amplicon sequencing could be superior than shotgun metagenomic sequencing for bacterial identification.(Tessler et al., 2017)

MinION (Oxford Nanopore Technologies(ONT), Oxford, UK), a nanopore sequencer, has many advantages that are suitable for metagenomic research. MinION produces nucleotide sequence data sequentially, enabling real-time analysis of the reads.(Marx, 2015) Moreover, MinION has further advantages including the low cost and small size of the device, simple and rapid library preparation, and capability of long-read sequencing.(Marx, 2015) Therefore, MinION might provide new opportunities in infectious disease diagnostics and surveillance.(Gardy and Loman, 2018) An increasing number of studies demonstrate that nanopore sequencing is capable of pathogen detection in various kinds of infections,(Greninger et al., 2015; Kilianski et al., 2015; Moon et al., 2018; Sanderson et al., 2018) and it has been successfully applied for the real-time surveillance Ebola and Zika outbreaks.(Faria et al., 2016; Quick et al., 2016) In addition, a number of studies have reported that bacterial identification can be achieved by 16S rDNA amplicon sequencing on MinION.(Mitsuhashi et al., 2017; Moon et al., 2018; Shin et al., 2016)

In the current study, we investigated if the MinION 16S amplicon sequencing is suitable for rapid bacterial detection directly from the cerebrospinal fluid (CSF) in bacterial meningitis patients.

2. Materials and methods

2.1. Clinical sample collection

We had been storing the initial pretreatment CSF of infectious meningoencephalitis patients who visited Seoul National University Hospital (SNUH) at −80 °C since June 2015. The stored CSF was obtained in parallel with the samples used for the conventional bacterial culture studies. The CSF samples obtained from six patients with confirmed bacterial meningitis cases were used for the experiments. In all of these cases, bacteria was isolated by standard culture methods from either the CSF or blood or from both (Table 1). In SNUH, MicroScan WalkAway (Siemens Healthcare Diagnostics, Germany) and VITEK2 (bioMérieux, France) are used for the identification of gram positive and gram negative bacteria, respectively. In Patient 1, bacteria were isolated from both blood and CSF culture, whereas bacteria were only grown from the CSF in Patient 2. In Patients 3–5, bacteria were only isolated from blood cultures but not from the CSF studies. Patient 6 was transferred to our hospital after 3 days of antibiotics treatment. Therefore, the blood and CSF culture studies performed at SNUH were all negative; however, the initial blood culture performed at the previous hospital isolated a bacterium (Table 1).

In the two prospective cases, bacterial meningitis was strongly suspected according to the clinical manifestation and the result of routine CSF analysis (patient 7 and 8; Table 1). Bacteria were isolated from the CSF culture in Patient 7, but no bacteria were isolated from the conventional diagnostic tests in Patient 8. The remaining initial pretreatment CSF was used for the experiments.

2.2. DNA extraction and 16s rDNA PCR

DNA was extracted from 200μℓ of the CSF using the PureLink genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). For each sample, PCR amplification of 16 s rDNA was performed using the Bacterial 16S rDNA PCR Kit (Takara, Tokyo, Japan) according to the manufacturer's instruction. In brief, the 16S rDNA primer mix (2.5 μℓ/25μℓ) (Takara, Tokyo, Japan) were added to the genomic DNA and PCR was performed. The PCR cycle condition was an initial denaturation at 94 °C for 1 min; 30 or 35 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C 1 min; and a final extension at 72 °C for 3 min. All PCR reactions were performed in a C1000™ thermal cycler (Bio-Rad, CA, USA). In every PCR reaction, negative control (distilled water) and positive control

Table 1
Patient demographics and the results of initial biochemical tests.

No.	Sex	Age	Diagnosis	BT	CRP	PCT	Initial CSF profile	CSF Prot (mg/dL)	CSF/Serum glucose	Blood Culture	CSF Culture
Retrospective cases											
1	M	65	Listeria monocytogenes meningitis	38.7	35.7	N/A	W50 (P62%, L8%, O28%)	385.4	0.06	L. monocytogenes	L. monocytogenes
2	M	77	Pseudomonas aeruginosa meningitis	38.2	12.1	N/A	W3744 (P90%, L9%)	201.8	0.2	(-)	P. aeruginosa
3	M	47	Klebsiella pneumoniae meningitis	39	35.3	6.63	W1980 (P54%, L2%, O44%)	292	0.01	K. pneumoniae	(-)
4	F	78	Streptococcus oralis meningitis	40.3	5.78	2.6	W9072 (P88%, O12%)	1082.4	0.04	S. oralis	(-)
5	M	79	Streptococcus agalactiae meningitis	38.8	0.6	N/A	W5500 (P99%, O1%)	414.1	0.19	Streptococcus agalactiae	(-)
6	F	57	Klebsiella pneumoniae meningitis with ventriculitis	38.5	12.4	15.51	W4464 (P92%, L2%, O6%)	152.9	0.17	(-), (K. pneumoniae: previous hospital)	(-)
Prospective cases											
7	F	36	Streptococcus viridans meningitis	38.6	11.9	N/A	W1548 (P90%,L8%,O2%)	131	0.02	S. viridans group	S. viridans group
8	F	83	Suspicious bacterial meningitis	39	25.5	N/A	W16704 (P91%, O9%)	900	0.01	(-)	(-)

Abbreviations: No, number; BT, body temperature; CRP, C-reactive protein; PCT, procalcitonin; CSF, cerebrospinal fluid; Prot, protein; M, male; F, female; N/A, not available; W, white blood cells (/mm^3); P, polymorphonuclear cells; L, lymphocytes; O, other cell.

(bacterial genomic DNA) were included. Products were electrophoresed on 1.5% agarose gel containing 0.05 μl/ml of RedSafe™ (Intron Biotechnology, Seoul, South Korea) and visualized using a Bio-Rad Gel Doc EZ Imager. When the PCR product of negative control demonstrated PCR positive band, contamination was suspected and PCR was performed again from the initial step.

2.3. MinION library preparation and sequencing

When the 16S PCR bands of the samples were seen on gel electrophoresis, sequencing libraries were prepared from the 16 s rDNA PCR products using Oxford Nanopore Sequencing kits (SQK-LSK108 for 1D sequencing, SQK-LSK308 for $1D^2$ sequencing, and SQK-RAD003 for rapid 1D sequencing, ONT, Oxford, UK). The input DNA was end-repaired and A-tailed using Ultra II End-prep enzyme (NEB, Hitchin, Hertfordshire, UK) incubated at 20 °C for 5 min and at 65 °C for 5 min. The end-prepped DNA was purified with AMPure XP (Beckman Coulter, High Wycombe, UK), and the DNA was eluted in nuclease-free water followed by ligation with 1D or $1D^2$ adapter using Blunt/TA Ligase Master Mix (NEB, Hertfordshire, UK) in room temperature for 10 min. The 1D or $1D^2$ adapter DNA purification was achieved with the Adapter Binding Buffer (ONT, Oxford, UK) using the magnetic stand, and the DNA library was eluted with the Elution Buffer (ONT, Oxford, UK). The pre-sequencing mix was loaded to flow cell FLO-MIN106 (for rapid 1D) or FLO-MIN107 (for 1D and $1D^2$) in a mix of Running Buffer with Fuel Mix and Library Loading Buffer (ONT, Oxford, UK). Finally, sequencing was performed for up to 3 h, and basecalling was performed using the MinKNOW software. The library preparation methods used in each case are shown in Table 2.

2.4. 16s rDNA analysis

During or after sequencing, the generated reads were analyzed by the cloud-based Metrichor/EPI2ME platform (Metrichor Ltd, Oxford, UK). The 16S analysis workflow of EPI2ME was used, which is designed to BLAST basecalled reads against the NCBI 16S bacterial database. Each read is classified as certain bacteria at the species level based on the % coverage and identity. The list of the bacteria was arranged in descending order according to the number of aligned reads. Then, the genus or species at the top of the list were regarded as the pathogen.

For the subgroup analysis of the reads in the retrospective cases, the reads were categorized according to the generated time after sequencing. We repeated the 16S analysis using the reads generated during the first 10 min, first 1 h, and total sequencing time, in each case.

3. Results

3.1. Pathogenic bacteria can be identified directly from CSF by 16S amplicon sequencing

In all of the retrospective cases, the 16S amplicon sequencing of CSF successfully detected the same bacteria that were isolated from conventional culture studies (Table 2). 16S amplicon sequencing detected the bacteria that were grown from the CSF culture studies in Patients 1 and 2. Meanwhile, bacterial meningitis was diagnosed based on the results of the blood culture in 4 patients (Patients 3 to 6). 16S amplicon sequencing detected the genomic sequences of each pathogenic bacterium from the CSF of these patients. The results of the 16S analyses in the representative cases are demonstrated in Fig. 1.

3.2. 16S amplicon sequencing was more sensitive than conventional diagnostic tests

16S amplicon sequencing was capable of bacterial identification using smaller amount of CSF than conventional tests. Normally, 1–3 ml of samples is required for conventional tests of bacterial detection,

Table 2
Sequencing data and the results of the 16S rRNA gene analyses at the Genus level and Species level.

No.	Culture studies result	PCR cycle	Library prep	Sequencing time	Total basecalled reads	16S analyzed reads	16S Aligned reads	Results (Genus)	Results (Species)
1	Listeria monocytogenes (B&C)	30	1D	1.5 h	99,380	99,167	65,600	Listeria 64,729 (98.7%)	L. monocytogenes 42,029 (64,1%), L. welshimeri 15,605 (23.8%)
2	Pseudomonas aeruginosa (C)	30	1D	3 h	400	400	18	Pseudomonas 17 (94.4%)	P. aeruginosa 17 (94.4%)
3	Klebsiella pneumoniae (B)	30	$1D^2$	2 h	40,043	40,002	13,728	Klebsiella 13,287 (96.8%)	K. pneunomiae 12,422 (90.5%)
4	Streptococcus oralis (B)	30	$1D^2$	3 h	14,274	14,272	3,204	Streptococcus 3,141 (98.0%)	S. oralis 1,870 (58.4%), S. mitis 774 (24,2%)
5	Streptococcus agalactiae (B)	30	1D	3 h	11,157	11,154	3,934	Streptococcus 3,917 (99.6%)	S. agalactiae 3,424 (87.0%)
6	Klebsiella pneumoniae (B)	30	$1D^2$	3 h	4,790	4,788	321	Klebsiella 142 (44.2%), Phyllobacterium 43 (13.4%)	K. pneunomiae 132 (41.1%), Phyllobacterium myrsinaceartum 43 (13.4%)
Additional Sequencing[†]									
2′	Pseudomonas aeruginosa (C)	35	Rapid 1D	3 h	14,896	14,886	550	Pseudomonas 515 (93.6%)	P. aeruginosa 414 (75.3%)
6′	Klebsiella pneumoniae (B)	35	Rapid 1D	3 h	31,572	31,554	6,344	Klebsiella 6,026 (95.0%)	K. pneunomiae 5,175 (81.6%)
Prospective cases									
7	Viridans Streptococci (B&C)	30	Rapid 1D	3 h	23,986	23,956	17,094	Streptococcus 16,010 (93.7%)	S. oralis 6,215 (36.4%), S. mitis 5,894 (34.5%)
8	No growth	30	$1D^2$	3 h	33,623	22,708	17,042	Streptococcus 16,743 (98.2%)	S. pneumoniae 13,283 (77.9%)

Abbreviations: No, number; Library prep, library preparation methods; h, hour.
[†] In Patients 2 and 6, PCR was repeated with increased amplification cycles (35 cycles) and additional sequencing was performed from these PCR products.

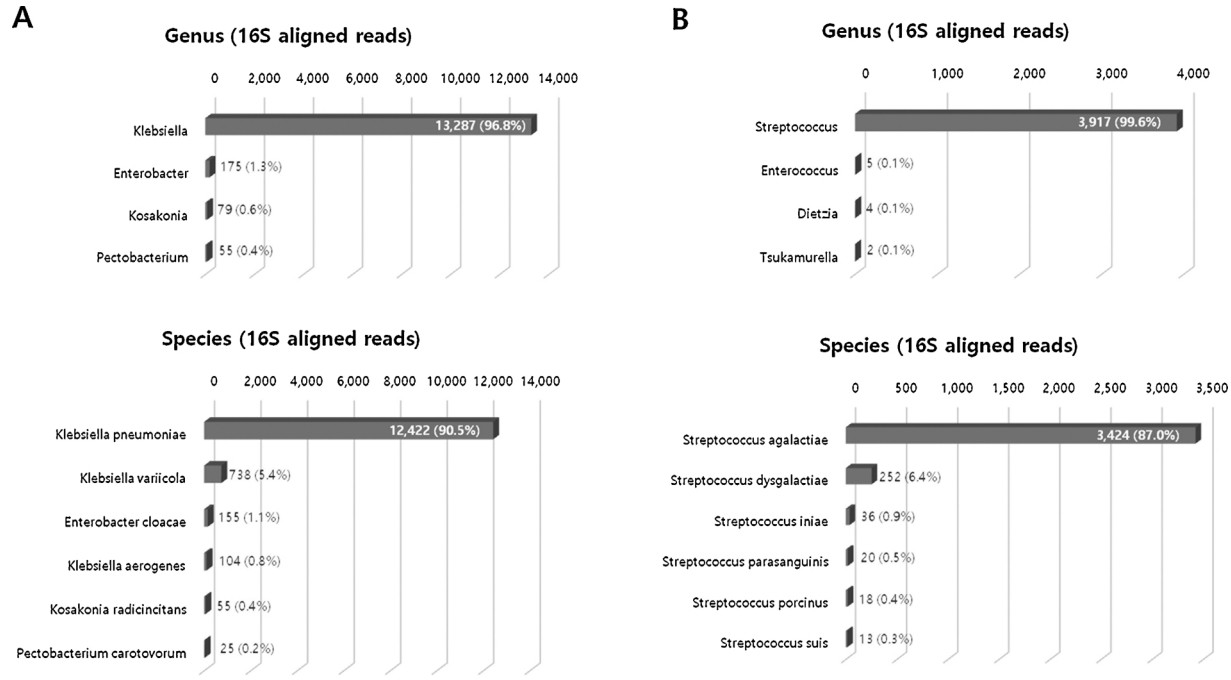

Fig. 1. Representative result of the 16S amplicon sequencing.
Panel (A) and (B) represents the data of patient 3 and patient 5, respectively. The 16S rDNA PCR performed on the CSF was positive so the sequencing library was generated from the PCR product in each case. After nanopore sequencing, the generated reads were aligned to bacterial sequences using the Epi2Me 16S analysis workflow. The top lists of the bacteria were regarded as the pathogen of the meningitis. Panel (A) displays that *Klebsiella* (genus), *K.pneumoniae* (species) were identified by 16S amplicon sequencing in patient 3. Panel (B) demonstrates that *Streptococcus* (genus), *S.agalactiae* (species) were detected by 16S amplicon sequencing in patient 5.

including gram stain and culture studies.(Connell et al., 2007; Gray and Fedorko, 1992) We used 200 μℓ of CSF for the DNA extraction, which was sufficient for multiple reactions of PCR and sequencing. Achieving the same result using smaller amount of samples suggests the superior sensitivity of the 16S amplicon sequencing over conventional tests.

16S amplicon sequencing detected bacterial sequences from CSF that showed negative results on culture studies. In Patient 3 to 5, bacteria were grown only from the blood but not from the CSF. 16S amplicon sequencing of the CSF has successfully detected the bacteria that were identical to those isolated in the blood culture in every patient (Table 2). Owing to the superior sensitivity of 16S amplicon sequencing, a definite diagnosis of bacterial meningitis would be made by proving that the pathogenic bacteria was present in the subarachnoid space. In Patient 6, the result of the CSF culture study performed at our hospital was negative, because the empirical antibiotics had been administered for 3 days. However, 16S amplicon sequencing was also capable of pathogen detection using this antibiotic-treated CSF.

3.3. MinION sequencing enabled significant reduction of the turnaround time

MinION sequencing enables real-time analysis of the reads; therefore, its ability to further reduce the turnaround time by shortening the sequencing time was investigated. In all of the cases, sequencing was performed for less than 3 h and reached the correct diagnosis. In Patients 1 and 3, we stopped sequencing after 1.5 h and 2 h, respectively, because the real-time analysis of the reads reached the correct answer (Table 2).

Next, we performed subgroup analysis of the reads according to its generated times to evaluate if a shorter time of sequencing, such as less than 3 h, would be sufficient for pathogen detection. The proportions (%) of the reads aligned to the correct bacterial species were very similar among the reads generated during the first 10 min, 1 h, and total sequencing time (1.5–3 h; Fig. 2) in every case. This finding implies that 10 min of sequencing would be sufficient for pathogen identification in some cases.

3.4. Sensitivity and turnaround time of 16S amplicon sequencing can be improved by protocol adjustment

Increasing the cycle number of PCR was helpful for the increment of reads aligned to the pathogenic bacteria in certain cases. In Patients 2 and 6, after the initial sequencing with 30-cycle PCR products, only 18 and 321 reads were aligned to bacterial 16 s rDNA sequences, respectively. Among them, only 17 and 132 reads were correctly aligned to the pathogenic bacterial species, respectively. When we performed sequencing with 35-cycle products, the number of reads correctly aligned to the pathogenic bacterial species increased to 414 and 5175, respectively. It also significantly reduced the proportion of the misaligned reads and made the results clearer. The total number of basecalled reads also increased in these cases, from 400 to 14,896 and from 4790 to 31,572, respectively (Table 2).

A rapid library preparation kit was also effective for bacterial identification, which could further reduce the turnaround time of the sequencing. The standard library preparation kits (SQK-LSK108 or SQK-LSK308) require nearly 2 h for the generation of sequencing libraries. (Runtuwene et al., 2018) Meanwhile, the rapid sequencing kit (SQK-RAD003) enables library preparation in less than 20 min. When performing the additional sequencing in Patients 2 and 6, we used the rapid sequencing kit for the library preparation and successfully identified the pathogenic bacteria in each sample.

3.5. Prospective application of MinION sequencing was successful

Next, we prospectively applied MinION 16S amplicon sequencing to patients who were highly suspected to have bacterial meningitis (Table 2). In Patient 7, 16S amplicon sequencing detected 16,010 reads aligned to *Streptococcus*. At the species level, *S. oralis* (6215 reads) were

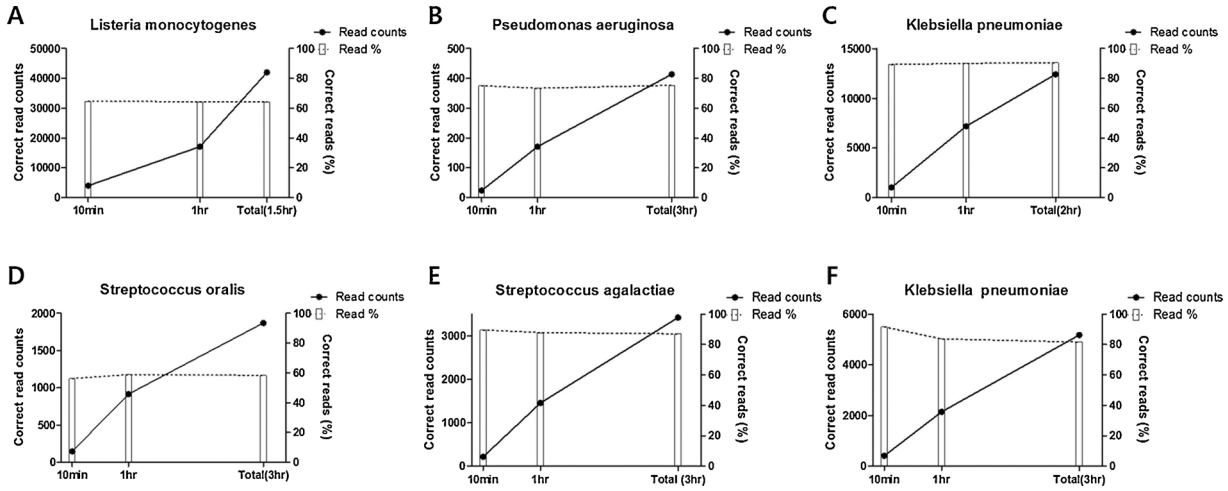

Fig. 2. Subgroup analysis of the sequencing reads according to its generated time.
Panels (A–F) represent the data of Patients 1-6. The number of reads that were correctly aligned the pathogen among the reads generated until 10 min, 1 h, and 1.5–3 h are indicated as the dots and solid lines on each panel. The percent of reads that were correctly aligned the pathogen among the reads generated until 10 min, 1 h, and 1.5–3 h are indicated as the bar graphs and the dotted lines on each panel. For example, in Patient 1, the numbers of reads aligned to Listeria monocytogenes among the reads generated until 10 min, 1 h, and 1.5 h were 4,015, 17,103, and 42,029, respectively. The percentages of reads aligned to Listeria monocytogenes among the reads generated until 10 min, 1 h, and 1.5 h were 64.7%, 64.3%, and 64.1%, respectively. In all cases, the percentages of reads aligned to the pathogen were equivalent regardless of the duration of the sequencing, suggesting that 10 min of sequencing would be equally informative as longer duration of sequencing for pathogen identification.

listed on the top followed by *S. mitis* (5894 reads). This finding should be interpreted as misalignments caused by sequence homology of the 16 s rDNAs between the two species (99% identical) instead of as co-infection with two species. On the next day, CSF culture study reported the growth of the viridans group streptococci, which was in accordance with the sequencing results. In Patient 8, 16S amplicon sequencing identified *Streptococcus pneumoniae* from the CSF of the patient. The bacterial culture studies were negative, and the pathogen was left undiagnosed by conventional diagnostic tests. Considering the clinical manifestations of the patient – accompanied by pansinusitis, high fever (39°C), severe CSF pleocytosis, and low CSF/serum glucose ratio – we believe it is plausible to diagnose this case as a by *S. pneumoniae* meningitis. To confirm the result, PCR for *S.pneumoniae* was performed from the CSF with the primer sequence and PCR conditions described in the previous study (Llull et al., 2006) and demonstrated a positive result (data not shown). These two cases demonstrate the excellent potential of MinION 16S amplicon sequencing for prospective application in bacterial meningitis.

4. Discussion

16S amplicon sequencing of the CSF was capable of rapid pathogen identification in bacterial meningitis. As it could detect the bacteria directly from the CSF, this method could significantly reduce the time required for making a diagnosis compared to conventional culture studies. Additionally, 16S amplicon sequencing was more sensitive than the conventional diagnostic tests. Moreover, MinION sequencing enabled further reduction of the turnaround time and the prospective application of the MinION sequencing was successful.

This is the first study to perform 16S amplicon deep sequencing from the CSF of bacterial meningitis patients. To date, a limited number of studies have applied 16 s rDNA analysis from CSF for the diagnosis of bacterial meningitis, but all of those studies applied Sanger sequencing methods.(Schuurman et al., 2004; Welinder-Olsson et al., 2007) Unfortunately, in a substantial portion of the cases (13/37 in Schuurman et al. and 14/32 in Wilinder-Olsson et al.), 16S sequencing failed to detect the bacteria that were isolated from the culture studies. This result is probably because the Sanger sequencing of the 16 s rDNA cannot be applied to polymicrobial specimens or contaminated specimens, because the presence of multiple templates results in superimposed reads that are generally uninterpretable.(Salipante et al., 2013) This problem can be solved by deep sequencing, which can be performed by MinION. Accordingly, we did not encounter any false negative results in this study.

16S amplicon sequencing enables bacterial detection directly from the CSF and will significantly reduce the time required for making diagnosis. Normally, bacterial culture studies require more than 2–5 days to isolate a bacterium.(Lagier et al., 2015) Rapid pathogen detection and early administration of antibiotics are crucial for the management of bacterial meningitis.(Proulx et al., 2005; van de Beek et al., 2012) When bacterial meningitis is suspected, empirical antibiotics, consisting of a third-generation cephalosporin plus vancomycin plus ampicillin (in old age), should be given as quickly as possible along with adjunctive dexamethasone.(Tunkel et al., 2004) Then, the antimicrobial regimen should be tailored when the CSF gram stain results are available. (Durand et al., 1993) When Gram-positive cocci are present, vancomycin plus a third-generation cephalosporin should be administered. If the Gram stain is negative, empirical antibiotics should be continued. (Durand et al., 1993) Thereafter, the antimicrobial regimen should be modified further according to the CSF culture and susceptibility results. Although 16 s rDNA analysis does not offer information about the antibiotic susceptibility of the bacteria, rapid bacterial identification will be extremely valuable for proper antibiotics adjustment.

16S amplicon sequencing was more sensitive than conventional culture studies. 16S amplicon sequencing of CSF was capable of bacterial detection with a much smaller volume of samples than culture studies and in cases in which the bacteria were only isolated from the blood culture but not from the CSF culture. 16S amplicon sequencing also identified the bacteria from antibiotic-treated samples. It has been reported that 16S sequencing can identify bacteria directly from clinical samples with better sensitivity than culture studies in various types of infections.(Chen et al., 2014; Jenkins et al., 2012; Pendleton et al., 2017; Woo et al., 2008) In bacterial meningitis, 16S sequencing was capable of bacterial detection in cases with negative results on culture studies, and it was particularly useful in antibiotic-treated samples, (Schuurman et al., 2004; Welinder-Olsson et al., 2007) which is in accordance with the current study.

MinION sequencing was helpful for further reducing the turnaround

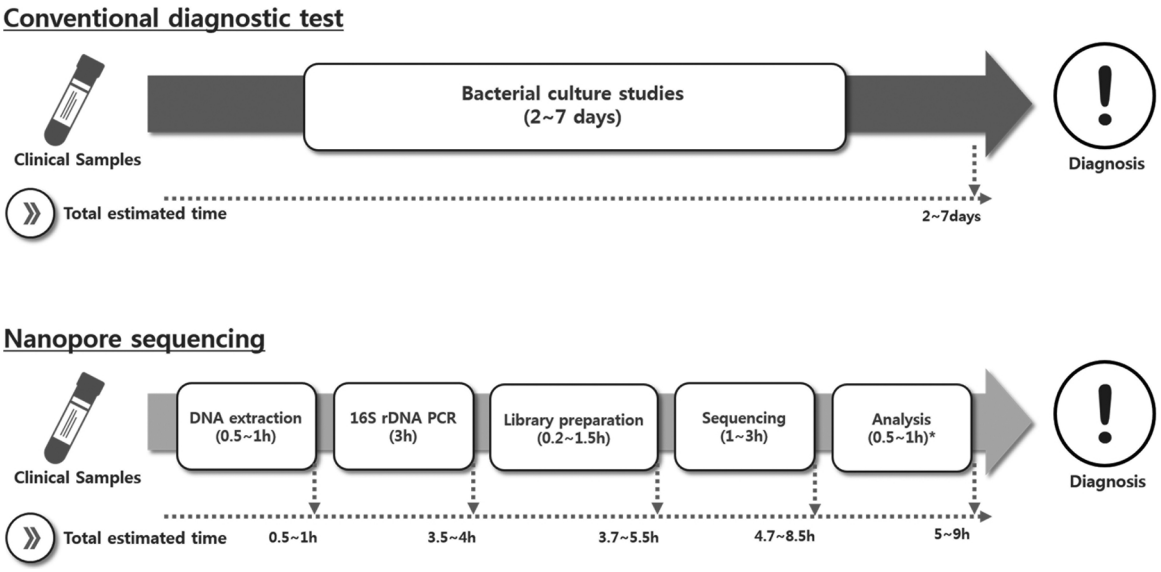

Fig. 3. Estimated turnaround time of each diagnostic methods.
The conventional bacterial culture studies usually require 2˜7 days for the identification of the bacteria. For the nanopore sequencing, DNA extraction, 16S rDNA PCR, library preparation, sequencing, and analysis should be performed and time required for each step is displayed. The cumulative time until the end of each step is demonstrated in the bottom, and total turnaround time is less than 9 h.
*Analysis of the reads can be performed in real-time, while sequencing is ongoing.

time of 16S amplicon sequencing. MinION enables real-time analysis of the reads; therefore, it is particularly useful for metagenomic pathogen detection.(Gardy and Loman, 2018; Greninger et al., 2015) Shortly after the public release of MinION sequencing high sequencing error rate was a concern for this technology. However, recent studies have demonstrated that nanopore sequencing can perform accurate sequencing (94–97% accuracy) of the microbial whole genome (Tyler et al., 2018) and the 16S rRNA gene (Kai et al., 2019). Also, deep sequencing can further get over the occurrence of misalignment during the metagenomic analysis. MinION has been successful for screening the Ebola outbreak (Quick et al., 2016) and the Zika outbreak (Faria et al., 2016). Additionally, 16 s rDNA analysis was stably performed by MinION in numerous studies (Kilianski et al., 2015; Moon et al., 2017; Shin et al., 2016). In all of our cases, sequencing for less than 3 h was sufficient for bacterial identification, and the real-time analysis of reads reached an answer before the sequencing was finished. The subgroup analysis of the reads suggests that in some cases, 10-minute sequencing would be enough for pathogen detection. Including the time required for DNA extraction, PCR, and library preparation, the total turnaround time was less than 9 h in every case (Fig. 3).

Further protocol adjustment would increase the sensitivity and reduce the turnaround time of the MinION sequencing. Increasing the number of PCR cycles was effective for amplifying the number of reads aligned to the pathogen. Similar strategy has been successfully applied in the metagenomic detection of viruses that are present in a very low titer within a sample.(Quick et al., 2017, 2016) Increasing PCR cycles might result in the generation of nonspecific PCR products,(Kebschull and Zador, 2015) but in our study, increasing up to 35 cycles worked efficiently for bacterial detection. MinION also has strength in its simple library preparation process. We have applied 3 different types of library preparation methods and they all worked well. Especially, the rapid sequencing kit was used in some of our cases, including one prospective case, and the library preparation took less than 20 min (10 min at best on the protocol) but successfully detected the pathogen. The rapid sequencing kit will be an optimal choice in prospective cases, and it will enable pathogen identification before the second dose of empirical antibiotics is given.

Prospective application of MinION 16S amplicon sequencing will be helpful for overcoming the current challenges of bacterial meningitis. (van de Beek, 2012) The diagnostic dilemmas of bacterial meningitis are present because clinical findings and CSF studies of the patients are not always diagnostic.(Brouwer et al., 2012) Multiplex PCR-based methods can be an effective approach for the rapid diagnosis of meningitis caused by common pathogens. But since bacterial meningitis is caused by hundreds of bacteria, pathogens not included in PCR panels will be missed. When bacteria can be identified rapidly by MinION 16S sequencing, it will detect all kinds of bacteria with a single diagnostic test and resolve the diagnostic dilemma early. The detrimental effects of delayed empirical antibiotics are widely known in the management of bacterial meningitis;(Brouwer et al., 2012; van de Beek et al., 2012) however, the importance of early change from empirical antibiotics to tailored antibiotics has not been investigated. If rapid bacterial identification is made by MinION, earlier switch to tailored antibiotics can be achieved and might lead to a better prognosis in bacterial meningitis. Also, antimicrobial resistance is a global concern in the management of infectious diseases.(Blair et al., 2015) An early switch to a tailored antibiotics will be helpful in reducing the emergence of multidrug resistant bacteria in the long term.

A limitation of our study is the small number of cases included in the experiments, and the majority were retrospective cases, mainly due to the low incidence of bacterial meningitis in our institution. Additionally, 16S sequencing does not provide information about the antibiotic susceptibility of the pathogens; therefore, culture studies should remain as the standard diagnostic test.

5. Conclusions

Nanopore 16S amplicon sequencing of CSF may have many advantages over conventional culture studies in the diagnosis of bacterial meningitis. This method can be faster and more sensitive than culture studies and can be performed with a smaller amount of sample and with antibiotic-treated samples. Because of its easy accessibility and small size, MinION possesses a huge potential to be utilized as a point-of-care test in clinical practice. Further studies are warranted to verify the merits of nanopore sequencing in the management of bacterial meningitis.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

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