〈Regular Article〉

Identification of the Causative Microorganism of Suspected Bacterial Meningitis by Exhaustive Analysis of the 16S rRNA Sequence - Achievements from a research experiment course by a 3rd year medical student -

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ABSTRACT Techniques based on nucleic acid amplification sometimes serve as complementary measures when bacterial culture alone cannot determine the cause of a suspected infection. Hereby presented is such a case of suspected bacterial meningitis. Although culture negative, real-time PCR targeting 16S rRNA genomic sequence yielded positive results; the amplified product was further subjected to exhaustive analysis of the 16S rRNA sequence, which revealed the sequence to be a member of the genus *Curtobacterium*, known as indigenous bacteria of the soil as well as a potential infective agent of humans. This result illustrates both the advantages and the limitations of nucleic acid amplification based techniques.

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INTRODUCTION

In the case of naturally sterile sites, bacterial cultures from specimens collected from the infectious site provide the most concrete proof of bacterial infection. However, the causative agents are not always identifiable; some microorganisms are difficult to culture, and the amount of available specimen is sometimes not abundant. Pediatric cases are often challenging due to the limited amount of available specimens. Techniques based on nucleic acid amplification sometimes serve as complementary measures when culture alone cannot determine the cause.

A 6-month-old boy was admitted due to suspected bacterial meningitis. Though clinical bacterial culture test failed to grow any microorganisims from the cerebrospinal fluid (CSF), experimental real-time PCR targeting bacterial 16S rRNA genomic sequence yielded positive signals. The amplified product from this real-time PCR was subjected to further exhaustive analysis.

This study is part of a research experiment course for a 3rd year medical student who carried out experiments in exhaustive analysis of the 16S rRNA sequence

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MATERIALS AND METHODS

Total nucleic acid to serve as template for real-time PCR was extracted from CSF using QIAmp MinElute Virus Spin Kit (QIAGEN GmbH, Hilden Germany), according to the manufacturer-provided instructions, with the exception of double-scaled volumes (starting material volume 400 μL) eluted into 100 μL.

Real-time PCR targeting bacterial 16S rRNA genomic sequence was carried out in triplicate on a CFX96 Real-time PCR System (Bio-Rad Laboratories, Inc., CA, USA). TaKaRa probe qPCR Mix (TaKaRa Bio, Inc, shiga, Japan) was used with primer/probes (Forward, 5' - AGTTTGATCMTGGCTCAG-3'; Reverse, 5' -GGACTACHAGGGTATCTAAT-3'; Probe, 5' -[FAM]-CGTATTACCGCGGCTGCTGGCAC-[BHQ1]-3') adopted from the literature[1]; this primer pair amplifies an approximately 800 bp region of the bacterial 16S rRNA sequence. The thermal condition for real-time PCR was: 45℃ for 15 minutes and 95℃ for 3 minutes, followed by 40 cycles of 95℃ for 8 seconds and 60℃ for 34 seconds.

Real-time PCR reactions yielding positive signals were collected and desalted and deproteinized ("cleaned-up") using Nucleospin PCR clean-up Gel extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), according to the manufacturer provided instructions, which was further run on a 1.2% agarose gel with 1× TAE buffer under 100[V] for approximately 30 min. The gel was post-stained with ethidium bromide and inspected with blue LED transilluminator (peak excitation wavelength 505 nm); the visualized band of approx. 800 bp was excised and subjected to Gel-extraction using the Nucleospin PCR clean-up Gel extraction kit.

A trace amount of the extracted product was further subjected to a second PCR using PrimeSTAR HS (premix) (TaKaRa Bio, Inc.) employing the same aforementioned primers (Forward, Reverse) at a final concentration of 0.2 μM in a 50 μL reaction. The thermal condition for this second PCR was: 95℃ for 15 seconds; followed by 40 cycles of 95℃ for 5 seconds, 55℃ for 5 seconds, and 72℃ for 50 seconds.

The second PCR product, "cleaned-up" using Nucleospin PCR clean-up Gel extraction kit, was subjected to bi-directional sequencing using primers Forward and Reverse, ordered from Eurofins Genomics, K.K. (Tokyo, Japan).

The final chromatogram traces were manually examined using FinchTV software (Geopiza, Inc., http://www.geospiza.com/finchtv), and the consensus sequence excluding the primer sequences was achieved. Sequences resembling the consensus sequence were searched using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The positions of the aforementioned oligonucleotide primers/probe plotted against 16S rRNA sequence of *E.coli* (accession number J01859.1) are illustrated in Fig. 1.

This study was approved by the institutional ethics board of Kawasaki Medical School (No. 2924).

RESULTS

One reaction of the triplicated real-time PCR yielded a positive signal, which was subjected to further analysis. Inspection of the chromatogram revealed the read using the Reverse primer to be unsatisfactory (contiguous overlapping peaks; data not shown). Thus, the final sequence was determined from consecutive mostly distinct peaks from the read using the Forward primer (517 bases, Fig. 2). The nucleotide BLAST search indicated that sequences deriving from *Curtobacterium* spp. were similar to this determined sequence. (Fig. 3).

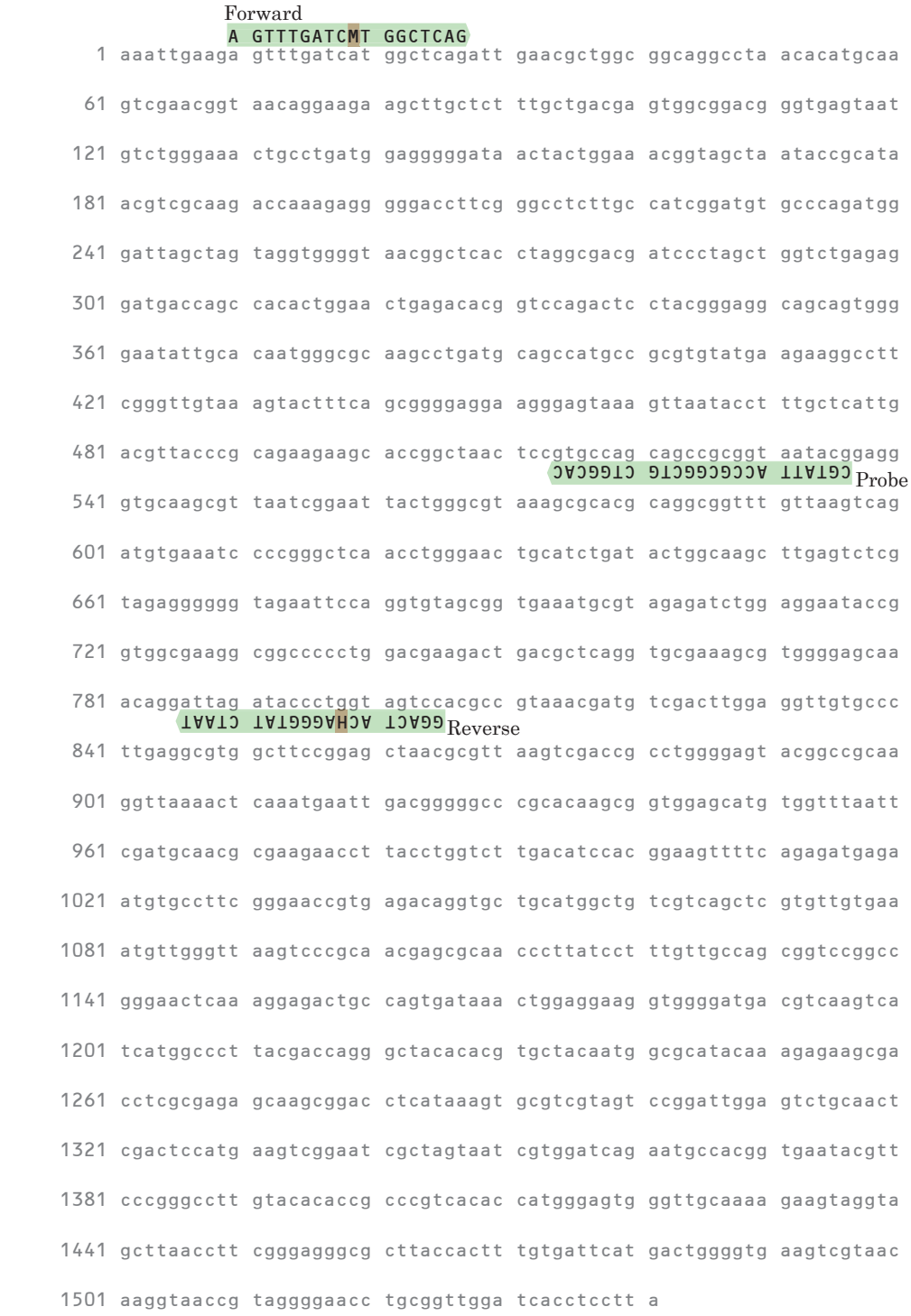
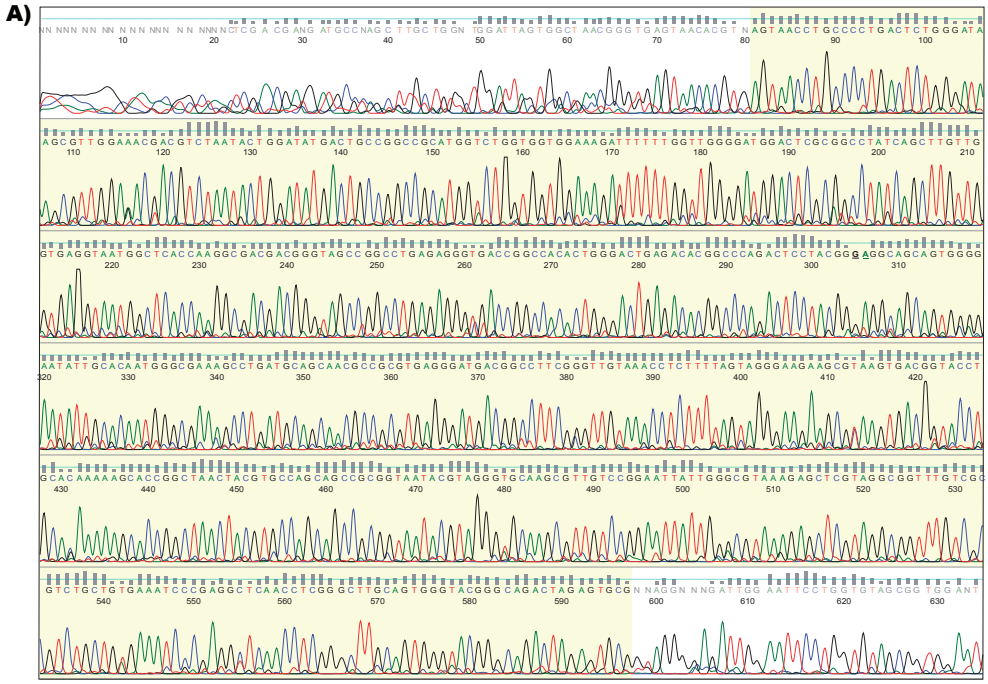


Fig. 1. The positions of the oligonucleotides employed in this study, plotted with E.coli 16S rRNA sequence (J01859.1)

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B)     1 GCGGGCGCAG CTTACCATGC AGTCGAACGA TGATGCCCAG CTTGCTGGGT GGATTAGTGG << KR780431.1
                                 AGTAACCTG CCCCTGACTC TGGGATAAGC GTTGGAAACG << Determined
      61 CGAACGGGTG AGTAACACGT GAGTAACCTG CCCCTGACTC TGGGATAAGC GTTGGAAACG

          ACGTCTAATA CTGGATATGA CTGCCGGCCG CATGGTCTGG TGGTGGAAAG ATTTTTTGGT
     121  ACGTCTAATA CTGGATATGA CTGCCGGCCG CATGGTCTGG TGGTGGAAAG ATTTTTTGGT

          TGGGGATGGA CTCGCGGCCT ATCAGCTTGT TGGTGAGGTA ATGGCTCACC AAGGCGACGA
     181  TGGGGATGGA CTCGCGGCCT ATCAGCTTGT TGGTGAGGTA ATGGCTCACC AAGGCGACGA

          CGGGTAGCCG GCCTGAGAGG GTGACCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC
     241  CGGGTAGCCG GCCTGAGAGG GTGACCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC

          TACGGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGAA AGCCTGATGC AGCAACGCCG
     301  TACGGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGAA AGCCTGATGC AGCAACGCCG

          CGTGAGGGAT GACGGCCTTC GGGTTGTAAA CCTCTTTTAG TAGGGAAGAA GCGTAAGTGA
     361  CGTGAGGGAT GACGGCCTTC GGGTTGTAAA CCTCTTTTAG TAGGGAAGAA GCGTAAGTGA

          CGGTACCTGC ACAAAAAGCA CCGGCTAACT ACGTGCCAGC AGCCGCGGTA ATACGTAGGG
     421  CGGTACCTGC AGAAAAAGCA CCGGCTAACT ACGTGCCAGC AGCCGCGGTA ATACGTAGGG

          TGCAAGCGTT GTCCGGAATT ATTGGGCGTA AAGAGCTCGT AGGCGGTTTG TCGCGTCTGC
     481  TGCAAGCGTT GTCCGGAATT ATTGGGCGTA AAGAGCTCGT AGGCGGTTTG TCGCGTCTGC

          TGTGAAATCC CGAGGCTCAA CCTCGGGCTT GCAGTGGGTA CGGGCAGACT AGAGTGCG
     541  TGTGAAATCC CGAGGCTCAA CCTCGGGCTT GCAGTGGGTA CGGGCAGACT AGAGTGCGGT

     601  AGGGGAGATT GGAATTCCTG GTGTAGCGGT GGAATGCGCA GATATCAGGA GGAACACCGA
     661  TGGCGAAGGC AGATCTCTGG GCCGTAACTG ACGCTGAGGA GCGAAAGCGT GGGGAGCGAA
     721  CAGGATTAGA TACCCTGGTA GTCCACGCCG TAAACGTTGG GCGCTAGATG TAGGGACCTT
     781  TCCACGGTTT CTGTGTCGTA GCTAACGCAT TAAGCGCCCC GCCTGGGGAG TACGGCCGCA
     841  AGGCTAAAAC TCAAAGGAAT TGACGGGGGC CCGCACAAGC GGCGGAGCAT GCGGATTAAT
     901  TCGATGCAAC GCGAAGAACC TTACCAAGGC TTGACATACA CCGGAAACGG CCAGAGATGG
     961  TTGCCCCCTT GTGGTCGGTG TACAGGTGGT GCATGGTTGT CGTCAGCTCG TGTCGTGAGA
    1021  TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTCGTTCTA TGTTGCCAGC GCGTTATGGC
    1081  GGGGACTCAT AGGAGACTGC CGGGGTCAAC TCGGAGGAAG GTGGGGATGA CGTCAAATCA
    1141  TCATGCCCCT TATGTCTTGG GCTTCACGCA TGCTACAATG GCCGGTACAA AGGGCTGCGA
    1201  TACCGTAAGG TGGAGCGAAT CCCAAAAAGC CGGTCTCAGT TCGGATTGAG GTCTGCAACT
    1261  CGACCTCATG AAGTCGGAGT CGCTAGTAAT CGCAGATCAG CAACGCTGCG GTGAATACGT
    1321  TCCCGGGCCT TGTACACACC GCCCGTCAAG TCATGAAAGT CGGTAACACC CGAAGCCGGT
    1381  GGCCTAACCC TTGTGGAAGA GCCGTCGAAG GGGATTCCGT
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Fig. 2. A) The chromatogram traces, called bases are displayed. Adopted peaks/base calls are highlighted. B) Determined 517 bp sequence (highlighted) aligned with *Curtobacterium sp*. CR27 16S rRNA gene, partial sequence (KR780431.1, 1420 bp). The triangle indicates the mismatch between the sequences.

Fig. 3. Nucleotide BLAST result of the determined sequence.

DISCUSSIONS

Real-time PCR followed by exhaustive analysis of the 16S rRNA sequence revealed a causative agent candidate, which classic clinical culture test could not. The revealed sequence was assumed to be of the genus *Curtobacterium*. Although reports of its isolation in humans are found infrequently in the literature [2, 3], members of this genus are known as bacteria indigenous to the soil.

This sequence was determined from a positive real-time PCR product. Since only one reaction among triplicates proved positive, the template concentration is expected to be extremely low, approximating a single template per reaction. The actual template copy number in PCR reactions under trace template concentrations approximating a single copy per reaction is known to follow the Poisson distribution; thus, theoretically, a triplicate reaction would not miss a single copy in 95% probability. Had it been a sequence of a "familiar" causative agent of pediatric meningitis, it should have served as supportive information suggesting the true pathogen. However, the analysis revealed a microorganism not necessarily known as a causative agent of pediatric meningitis. Though there are a few reports of bacteria of the same genus to having infected humans, and taking into consideration the fact that the microorganism is a bacteria indigenous to the environment, whether the microorganism was the true cause or an adventitious introduction to the specimen (contamination) from the environment, via airborne particles, cannot be determined merely from our results.

This might, in part, indicate the characteristics of nucleic acid based methods against culture in the detection of traces of microorganisms. The extreme low detection limit of nucleic acid amplification, which is capable of detecting a single copy of the sequence of interest, under optimized conditions, is apparently an advantage. However, given the possibility of detecting trace contaminants, a detection limit too low would turn into a disadvantage; if the detection target is ubiquitous, the chance of adventitious contamination rises. Thus, interpretation of the results in the clinical context is crucial in the application of such techniques.

This study has the following limitation: Clinical bacterial culture/isolation test could not prove any bacteria from the identical specimen. Furthermore, the initial bacterial load estimate being extremely low, contamination of the specimen during all procedures cannot be denied; however, even multiplicated negative controls cannot necessarily suggest nor deny trace contamination by a single adventitious bacterium.

In conclusion, exhaustive analysis of 16S rRNA sequence proved its advantages over bacterial culture, as well as disclosing its limitations, by successively revealing the sequence of a candidate causative agent which was better known as bacteria indigenous to the environment.

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CONFLICT OF INTEREST

The authors have no conflicts of interest directly relevant to the content of this article.

REFERENCES

1) Zucol F, Ammann RA, Berger C, *et al*: Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. J Clin Microbiol 44: 2750-2759, 2006

2) Funke G, Aravena-Roman M, Frodl R: First description

of *Curtobacterium* spp. isolated from human clinical specimens. J Clin Microbiol 43: 1032-1036, 2005

3）Francis MJ, Doherty RR, Patel M, *et al*: *Curtobacterium flaccumfaciens* septic arthritis following puncture with a Coxspur Hawthorn thorn. J Clin Microbiol 49: 2759-2760, 2011