$\langle \text{Regular Article} \rangle$

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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Key words: 16S rRNA, Exhaustive Analysis, Curtobacterium

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 realtime 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 3^{rd} 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 realtime 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 μ L) eluted into 100 μ L.

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-[BHO1]-3') adopted from the literature¹; this primer pair amplifies an approximately 800 bp region of the bacterial 16S rRNA sequence. The thermal condition for real-time PCR was: 45 °C for 15 minutes and 95°C 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 $1 \times$ 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 Gelextraction 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 μ M in a 50 μ L reaction. The thermal condition for this second PCR was: 95°C for 15 seconds; followed by 40 cycles of 95°C for 5 seconds, 55°C for 5 seconds, and 72°C 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.

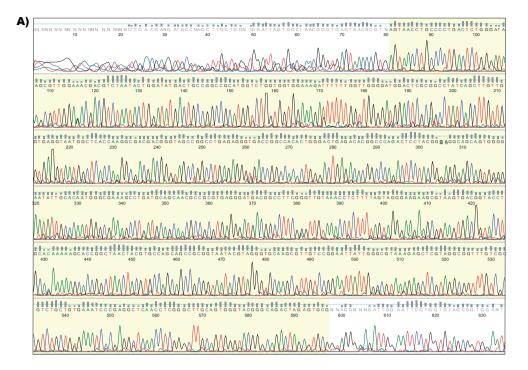
This study was approved by the institutional ethics board of Kawasaki Medical School (No. 2924).

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).

	A	orward GTTTGATCMT				
1	aaattgaaga	gtttgatcat	ggctcagatt	gaacgctggc	ggcaggccta	acacatgcaa
61	gtcgaacggt	aacaggaaga	agcttgctct	ttgctgacga	gtggcggacg	ggtgagtaat
121	gtctgggaaa	ctgcctgatg	gagggggata	actactggaa	acggtagcta	ataccgcata
181	acgtcgcaag	accaaagagg	gggaccttcg	ggcctcttgc	catcggatgt	gcccagatgg
241	gattagctag	taggtggggt	aacggctcac	ctaggcgacg	atccctagct	ggtctgagag
301	gatgaccagc	cacactggaa	ctgagacacg	gtccagactc	ctacgggagg	cagcagtggg
361	gaatattgca	caatgggcgc	aagcctgatg	cagccatgcc	gcgtgtatga	agaaggcctt
421	cgggttgtaa	agtactttca	gcggggagga	agggagtaaa	gttaatacct	ttgctcattg
481	acgttacccg	cagaagaagc	accggctaac	tccgtgccag O¥O99LO	cagccgcggt 910990900¥	aatacggagg 11¥193 Probe
541	gtgcaagcgt	taatcggaat	tactgggcgt			
601	atgtgaaatc	cccgggctca	acctgggaac	tgcatctgat	actggcaagc	ttgagtctcg
661	tagagggggg	tagaattcca	ggtgtagcgg	tgaaatgcgt	agagatctgg	aggaataccg
721	gtggcgaagg	cggccccctg	gacgaagact	gacgctcagg	tgcgaaagcg	tggggagcaa
781	acaggattag	ataccctggt L¥L999¥HJ¥	agtccacgcc	gtaaacgatg e	tcgacttgga	ggttgtgccc
841	ttgaggcgtg				cctggggagt	acggccgcaa
901	ggttaaaact	caaatgaatt	gacgggggcc	cgcacaagcg	gtggagcatg	tggtttaatt
961	cgatgcaacg	cgaagaacct	tacctggtct	tgacatccac	ggaagttttc	agagatgaga
1021	atgtgccttc	gggaaccgtg	agacaggtgc	tgcatggctg	tcgtcagctc	gtgttgtgaa
1081	atgttgggtt	aagtcccgca	acgagcgcaa	cccttatcct	ttgttgccag	cggtccggcc
1141	gggaactcaa	aggagactgc	cagtgataaa	ctggaggaag	gtggggatga	cgtcaagtca
1201	tcatggccct	tacgaccagg	gctacacacg	tgctacaatg	gcgcatacaa	agagaagcga
1261	cctcgcgaga	gcaagcggac	ctcataaagt	gcgtcgtagt	ccggattgga	gtctgcaact
1321	cgactccatg	aagtcggaat	cgctagtaat	cgtggatcag	aatgccacgg	tgaatacgtt
1381	cccgggcctt	gtacacaccg	cccgtcacac	catgggagtg	ggttgcaaaa	gaagtaggta
1441	gcttaacctt	cgggagggcg	cttaccactt	tgtgattcat	gactggggtg	aagtcgtaac
1501	aaggtaaccg	taggggaacc	tgcggttgga	tcacctcctt	а	

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



B)	1	GCGGGCGCAG	CTTACCATGC	AGTCGAACGA	TGATGCCCAG	CTTGCTGGGT	GGATTAGTGG	<< KR780431.1
	61	CGAACGGGTG	AGTAACACGT		CCCCTGACTC CCCCTGACTC			< Determined
	121	ACGTCTAATA ACGTCTAATA	CTGGATATGA CTGGATATGA	CTGCCGGCCG CTGCCGGCCG	CATGGTCTGG CATGGTCTGG	TGGTGGAAAG TGGTGGAAAG	ATTTTTTGGT ATTTTTTGGT	
	181	<mark>TGGGGATGGA</mark> TGGGGATGGA	CTCGCGGCCT CTCGCGGCCT	ATCAGCTTGT ATCAGCTTGT	TGGTGAGGTA TGGTGAGGTA	ATGGCTCACC ATGGCTCACC	AAGGCGACGA AAGGCGACGA	
	241	CGGGTAGCCG CGGGTAGCCG	GCCTGAGAGG GCCTGAGAGG	GTGACCGGCC GTGACCGGCC	ACACTGGGAC ACACTGGGAC	TGAGACACGG TGAGACACGG	CCCAGACTCC CCCAGACTCC	
	301				AATGGGCGAA AATGGGCGAA			
	361	<mark>CGTGAGGGAT</mark> CGTGAGGGAT	GACGGCCTTC GACGGCCTTC	GGGTTGTAAA GGGTTGTAAA	CCTCTTTTAG CCTCTTTTAG	TAGGGAAGAA TAGGGAAGAA	GCGTAAGTGA GCGTAAGTGA	
	421	CGGTACCTGC CGGTACCTGC	AČAAAAAGCA AGAAAAAGCA	CCGGCTAACT CCGGCTAACT	ACGTGCCAGC ACGTGCCAGC	AGCCGCGGTA AGCCGCGGTA	ATACGTAGGG ATACGTAGGG	
		TGCAAGCGTT	GICCGGAATT	ATTGGGCGTA	AAGAGCTCGT	AGGCGGTTTG	ICGCGICIGC	
	481	TGCAAGCGTT	GICCGGAAII	AIIGGGCGIA	AAGAGCICGI	AGGCGGIIIG		
		TGTGAAATCC	CGAGGCTCAA	CCTCGGGCTT	GCAGTGGGTA GCAGTGGGTA	CGGGCAGACT	AGAGTGCG	
	541	<mark>TGTGAAATCC</mark> TGTGAAATCC	CGAGGCTCAA CGAGGCTCAA	CCTCGGGCTT CCTCGGGCTT	GCAGTGGGTA GCAGTGGGTA	CGGGCAGACT CGGGCAGACT	AGAGTGCG AGAGTGCGGT	
	541 601	TGTGAAATCC TGTGAAATCC AGGGGAGATT	CGAGGCTCAA CGAGGCTCAA GGAATTCCTG	CCTCGGGCTT CCTCGGGCTT GTGTAGCGGT	GCAGTGGGTA	CGGGCAGACT CGGGCAGACT GATATCAGGA	AGAGTGCG AGAGTGCGGT GGAACACCGA	
	541 601 661	TGTGAAATCC TGTGAAATCC AGGGGGAGATT TGGCGAAGGC	CGAGGCTCAA CGAGGCTCAA GGAATTCCTG AGATCTCTGG	CCTCGGGCTT CCTCGGGCTT GTGTAGCGGT GCCGTAACTG	GCAGTGGGTA GCAGTGGGTA GGAATGCGCA	CGGGCAGACT CGGGCAGACT GATATCAGGA GCGAAAGCGT	AGAGTGCG AGAGTGCGGT GGAACACCGA GGGGAGCGAA	
	541 601 661 721	TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA	CGAGGCTCAA CGAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA	CCTCGGGCTT CCTCGGGCCTT GTGTAGCGGT GCCGTAACTG GTCCACGCCG	GCAGTGGGTA GCAGTGGGTA GGAATGCGCA ACGCTGAGGA	CGGGCAGACT CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG	AGAGTGCG AGAGTGCGGT GGAACACCGA GGGGAGCGAA TAGGGACCTT	
	541 601 661 721 781	TGTGAAATCC TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTT	CGAGGCTCAA CGAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA	CCTCGGGCTT CCTCGGGCTT GTGTAGCGGT GCCGTAACTG GTCCACGCCG GCTAACGCAT	GCAGTGGGTA GCAGTGGGTA GGAATGCGCA ACGCTGAGGA TAAACGTTGG	CGGGCAGACT CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGGAG	AGAGTGCG AGAGTGCGGT GGAACACCGA GGGGAGCGAA TAGGGACCTT TACGGCCGCA	
	541 601 661 721 781 841	TGTGAAATCC TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTT AGGCTAAAAC	CGAGGCTCAA CGAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA TCAAAGGAAT	CCTCGGGCTT CCTCGGGCTT GTGTAGCGGT GCCGTAACTG GTCCACGCCG GCTAACGCAT TGACGGGGGC	GCAGTGGGTA GCAGTGGGTA GGAATGCGCA ACGCTGAGGA TAAACGTTGG TAAGCGCCCC	CGGGCAGACT CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGGAG GGCGGAGCAT	AGAGTGCG AGAACACCGGT GGGAACACCGA GGGGAGCGAA TAGGGACCTT TACGGCCGCA GCGGATTAAT	
	541 601 661 721 781 841 901	TGTGAAATCC TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTT AGGCTAAAAC TCGATGCAAC	CGAAGGCTCAA CGAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA TCAAAGGAAT GCGAAGAACC	CCTCGGGCTT GTGTAGCGGT GCCGTAACGG GTCCACGCCG GCTAACGCAT TGACGGGGGC TTACCAAGGC	GCAGTGGGTA GCAATGCGCA ACGCTGAGGA TAAACGTTGG TAAGCGCCCC CCGCACAAGC	CGGGCAGACT CGGCCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGGAG GGCCGAGCAT CCGGAAACGG	AGAGTGCG AGAGTGCGGT GGAACACCGA GGGAGCGAA TAGGGACCTT TACGGCCGCA GCGATTAAT CCAGAGATGG	
	541 601 661 721 781 841 901 961 1021	TGTGAAATCC TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTA AGGCTAAAAC TCGATGCAAC TTGCCCCTT TGTTGGGTTA	CGAAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA TCGAAGGAAT GCGAAGAACC GTGGTCGGTG AGTCCCGCAA	CCTCGGGCTT CCTCGGGCTT GCCGTAACTG GTCCACGCCG GCTAACGCAT TGACGGGGGC TACAGGTGGT CGAGCGCAAC	GCAGTGGGTA GCAGTGGGTA GGAATGCGCA ACGCTGAGGA TAAACGTTGG TAAGCGCCCC CCGCACAAGC TTGACATACA GCATGGTTGT CCTCGTTCTA	CGGGCAGACT CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGGAG GGCGGAGCAT CCGGAAACGG CGTCAGCTCG TGTTGCCAGC	AGAGTGCG AGACACCGA GGGACACCGA TAGGGACCTT TACGGCCGCA CCGGATTAAT CCAGAGATGG GCCGTATGGC	
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	541 601 721 781 901 961 1021 1081 1141 1201	TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTAGA TCCACGGTAAAC TCGATGCAAC TGGTGGGTA GGGGACTCAT TCATGCCCCT TACCGTAAGG	CGAAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA TCAAAGGAAT GCGAAGAACC GTGGTCCGGTG AGTCCCGCAA AGGAGACTGC TATGTCTTGG TGGAGCGAAT	CCTCGGGGCTT CCCCCGGGCTT CCCCCCGCG GCCAACCGG GCCAACGCGG GCTAACGCAG TACCAGGGGC TACCAGGGGGC TACCAGGGGGC CGGGGCCAAC CGGGGCCAAC GCTTCACGCA	GCAGTGGGTA GCAGTGGGTA ACGCTGAGGA TAAACGTTGG TAAGCGCCCC CCGCACAAGC TTGACATACA GCATGGTTGT CCTCGTTCTA TCGGAGGAAG TGCTACAATG CGGTCTCAGT	CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGAGCAT CCGGAAACGG CGTCAGCTCG TGTGCCAGC GTGGGGATGA GCCGGTACAA TCGGATTGAG	AGAGTGCG AGACACCGA GGGAACACCGA TAGGGACCTA TAGGGACCTA CCAGAGATGA GCGATTAAT CCAGAGATGA GCGTTATGGC CGTCAAATCA AGGGCTGCGA GTCTGCAACT	
	541 601 661 721 781 841 901 961 1021 1081 1141 1201 1261	TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTT AGGCTAAAAC TCGACGATCCAC TGCCCCTT TGTTGGGTTA GGGACTCAT TCATGCCCCT TACCGTAAGG CGACCTCATG	CGAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA TCAAAGGAAT GCGAAGAACC GTGGTCGGTG AGTCCCGCAA AGGAGACTGC TGGAGCGAAT AAGTCGGAGT	CTTCGGGCTT GCGTAGCGGT GCCGTAACTG GTCCACGCCG GCTAACGGGGC TTACAGGGGGC TTACAGGTGGT CGAGCGCAAC CGGGGTCAAC GCTTACAGCA CCCAAAAAGC CGCTAGTAAT	GCAGTGGGTA GCAGTGGGTA ACGCTGAGGA TAAACGTTGG TAGCGCCCC CCGCACAAGC TTGACATACA GCATGGTTGT CCTCGTTCTA TCGGAGGAAG TGCTACAATG CGGTCTCAGT	CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGGAG GCCGGAACGG CGTCAGCTCG TGTTGCCAGC GTGGGGATGA CCGGTACAA CGGATTGAG CAACGCTGCG	AGAGTGCG AGACACCGA GGGACCGAA TAGGACCTT TACGGCCGCA GCGGATTAAT CCAGAGATGG GCGTTATGGC CGTCAAATCA AGGCTGCGA GTCTGCAACT GTGAATACGT	
	541 601 661 721 781 841 901 961 1021 1081 1141 1201 1261 1321	TGTGAAATCC AGGGGAGATT TGGCGAAGGC CAGGATTAGA TCCACGGTTT AGGCTAAAAC TGGCGCCCT TGTTGGGTTA GGGGACTCAT TCATGCCCCT TACCGTAAGG CGACCTCATG TCCCGGGCCT	CGAGGCTCAA GGAATTCCTG AGATCTCTGG TACCCTGGTA CTGTGTCGTA TCAAAGGAAT GCGAAGAACC GTGGTCGGTG AGTCCCGCAA AGGAGACTGC TGGAGCGAAT AAGTCGGAGT	CCTCGGGGCTT GCCGTAGCGGT GCCGTAACTG GTCACGCCG GCTAACGCCG TACCAGGGGC TACCAAGGTGGT CGAGCGCAAC CGGGGTCAAC GCTCACGCA CCCAAAAAGC CGCTAGTAAT GCCCGTCAAG	GCAGTGGGTA GCAGTGGGGTA ACGCTGAGGA TAAACGTGG CCGCACAAGC TTGACATACA CCATGGTGT CCTCGTTCTA TCGGAGGAAG TGCTACAATG CGGTCTCAGT CGCAGATCAG TCATGAAAGT	CGGGCAGACT GATATCAGGA GCGAAAGCGT GCGCTAGATG GCCTGGGGAG GCCGGAACGG CGTCAGCTCG TGTTGCCAGC GTGGGGATGA CCGGTACAA CGGATTGAG CAACGCTGCG	AGAGTGCG AGACACCGA GGGACCGAA TAGGACCTT TACGGCCGCA GCGGATTAAT CCAGAGATGG GCGTTATGGC CGTCAAATCA AGGCTGCGA GTCTGCAACT GTGAATACGT	

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.

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		Description		score	score	cover	value	ldent	Accession	
		Curtobacterium sp. strain HA15-31 16S ribosomal RNA gene	partial sequence	950	950	100%	0.0	99%	MH769132.1	
		Curtobacterium sp. strain 2.165 ribosomal RNA gene, partial	sequence	950	950	100%	0.0	99%	MG664687.1	
		Curtobacterium albidum strain RTO 16S ribosomal RNA gen		950	950	100%	0.0	99%	MK014287.1	
		Curtobacterium citreum strain G27 165 ribosomal RNA gene		950	950	100%	0.0	99%	MH930061.1	
		Curtobacterium sp. strain 864.2.16S ribosomal RNA gene, pa		950 950	950 950	100%	0.0	99% 99%	MH777911.1 MF959447.1	
		Curtobacterium citreum strain GX 35 16S ribosomal RNA ge Curtobacterium citreum strain GX 32 16S ribosomal RNA ge		950 950	950 950	100%	0.0	99% 99%	MF959447.1 MF959443.1	
		Curtobacterium citreum strain GX 25 16S ribosomal RNA ge		950	950	100%	0.0		MF959443.1	
		Curtobacterium citreum strain GX 21 16S ribosomal RNA ge		950	950	100%	0.0	99%	MF959442.1	
		Curtobacterium citreum strain GX_18_16S ribosomal RNA ge	ne, partial sequence	950	950	100%	0.0	99%	MF959441.1	
		Curtobacterium citreum strain GX_11_16S ribosomal RNA gen	ne, partial sequence	950	950	100%	0.0	99%	MF959433.1	
		Uncultured bacterium clone 70.sp.AR 16S ribosomal RNA ge		950	950	100%	0.0		KX34450 <u>3.1</u>	
		Curtobacterium citreum strain VN2013-30.16S ribosomal RN		950	950	100%	0.0		<u>KX449259.1</u>	
		Uncultured bacterium clone HL2416S ribosomal RNA gene, Uncultured bacterium clone AL1316S ribosomal RNA gene,		950 950	950 950	100%	0.0	99%	KX212146.1 KX212113.1	
		Curtobacterium sp. strain FD5-15 16S ribosomal RNA gene.		950	950	100%	0.0		KX914495.1	
		Curtobacterium sp. SG33 16S ribosomal RNA gene, partial s		950	950	100%	0.0	99%	KR856488.1	
-	2	Uncultured Micrococcineae bacterium gene for 16S rRNA, par		950	050	100%		99%		-
		Curtobacterium citreum strain CC-88221 165 ribosomal RNA		950 950	950 950	100%	0.0		AB11460E1 AY9619861	
		Curtobacterium citreum strain DSM 20528 165 ribosomal RN		950	950	100%	0.0		NR 026136.1	
		Curtobacterium sp. IIL-Nv6 16S ribosomal RNA gene, partial		946	946	100%	0.0		GU37304).1	
		Curtobacterium sp. strain a113164 16S ribosomal RNA gene	partial sequence	944	944	100%	0.0	99%	MK280704.1	
		Curtobacterium sp. strain ATRS4.16S ribosomal RNA gene, p	artial sequence	944	944	100%	0.0	99%	MK25505'.1	
		Curtobacterium sp. strain FAW5-3 16S ribosomal RNA gene,		944	944	100%	0.0		MK248125.1	
		Curtobacterium sp. strain QS16-27 16S ribosomal RNA gene		944	944	100%	0.0	99%	MH769451.1	
		Curtobacterium sp. strain QS18-26.16S ribosomal RNA gene		944	944	100%	0.0	99%	MH769453.1	
		Curtobacterium sp. strain QS16-24.16S ribosomal RNA gene Curtobacterium sp. strain QS16-23.16S ribosomal RNA gene		944 944	944 944	100%	0.0	99% 99%	MH769451.1 MH769453.1	
		Curtobacterium sp. strain QS16-1916S ribosomal RNA gene		944	944	100%	0.0	99%	MH769447.1	
		Curtobacterium sp. strain QS16-14 16S ribosomal RNA gene		944	944	100%	0.0	99%	MH769443.1	
		Curtobacterium sp. strain QS16-13 16S ribosomal RNA gene		944	944	100%	0.0	99%	MH769442.1	
		Curtobacterium sp. strain CS16-19 16S ribosomal RNA gene	, partial sequence	944	944	100%	0.0	99%	MH769383.1	
		Curtobacterium sp. strain CS16-9 16S ribosomal RNA gene,	partial sequence	944	944	100%	0.0	99%	MH769373.1	
		Curtobacterium sp. strain GA15-20 165 ribosomal RNA gene		944	944	100%	0.0		MH769093.1	
		Curtobacterium sp. strain QA15-16 16S ribosomal RNA gene		944	944	100%	0.0		MH769092.1	
		Curtobacterium sp. strain QA15-816S ribosomal RNA gene, Curtobacterium oceanosedimentum strain SAK116S ribosor		944 944	944 944	100% 100%	0.0 0.0	99% 99%	MH769085.1 MF949056.1	
		Curtopacterium oceanosedimentum strain SAKT 165 ribosor Curtopacterium citreum strain PgBE223 165 ribosomal RNA.		944 944	944 944	100%	0.0		MH144291.1	
		Curtobacterium citreum strain SDSB9 16S ribosomal RNA ge		944	944	100%	0.0		MG892862.1	
		Curtobacterium citreum strain ODODD 100 housemannen ge		944	944	100%	0.0		MG705713.1	
		Curtobacterium sp. strain B4 16S ribosomal RNA gene, partia		944	944	100%	0.0	99%	MG234433.1	
		Curtobacterium oceanosedimentum strain SOGA 316S ribo	somal RNA gene, partial sequence	944	944	100%	0.0	99%	MH216663.1	
		Curtobacterium sp. strain R1.16S ribosomal RNA gene, parti		944	944				<u>MH141447.1</u>	
		Curtobacterium sp. strain DQMS04 168 ribosomal RNA gene		944	944				KX925565.1	
		Curtobacterium sp. strain MadaFrogSkinBac.DB-181916S ri		944	944				MF526831.1	
		Curtobacterium sp. strain MadaFrogSkinBac.DB- 740 168 rib Curtobacterium sp. strain MadaFrogSkinBac.DB- 2681 168 ri		944 944	944 944	100% 100%	0.0		MF524986.1 MF524969.1	
		Curtopacterium sp. strain MadaFrogSkinBac.DB-2681165 n Curtobacterium sp. strain MadaFrogSkinBac.DB-1276165 ni		944 944	944 944	100%	0.0		MF524969.1 MF524751.1	
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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.

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