IDENTIFICATION OF THE ATYPICAL BACTERIAL STRAIN *STREPTOCOCCUS INTERMEDIUS* THAT CAUSED BRAIN ABSCESS IN THE PATIENT USING SANGER SEQUENCING OF THE 16S RRNA GENE FROM THE DNA EXTRACTED FROM A PUS SAMPLE

Gordukova MA[1] ✉, Divilina YuV[1], Mishukova OV[2], Galeeva EV[1], Prodeus AP[1], Filipenko ML[2]

[1] Clinical Diagnostic Laboratory,
Speransky Children's Clinical Hospital No. 9, Moscow, Russia

[2] Laboratory of Pharmacogenomics,
Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia

In this article we present a clinical case of brain abscess in a girl aged 14 years and 11 months caused by a pathogen that could not be identified by routine microbiological testing. Before admission and during her stay in the hospital, the teenager did not have fever. Diagnosis and treatment were impeded by allergic responses to a wide range of antibiotics. The patient underwent three surgical interventions. Pus culture was performed 4 times, showing no growth. A PCR assay was run twice, but both times the results came out negative. Therefore, a decision was made to amplify and Sanger-sequence the 16S rRNA gene from the DNA extracted from patient's pus. BLAST showed a 99 % homology of the obtained nucleotide sequence to the sequence of the 16S rRNA gene of *Streptococcus intermedius* (strain ChDC B589, KF733728.1) which had been previously shown to play a role in brain abscess development. Treatment **ex juvantibus** against the pathogen was started before sequencing results were available. The patient responded positively, the symptoms were alleviated and the condition improved. Thus, we conclude that in some cases sequencing may be the only diagnostic technique capable of identifying the pathogen.

Keywords: brain abscess, *Streptococcus intermedius*, 16S rRNA, Sanger sequencing, laboratory diagnosis

✉ **Correspondence should be addressed:** Maria Gordukova
Shmitovsky proezd, d. 29, Moscow, Russia, 123317; ma.gordukova@dgkb-9.ru

Received: 04.07.2017 **Accepted:** 04.08.2017

ИДЕНТИФИКАЦИЯ НЕТИПИЧНОГО БАКТЕРИАЛЬНОГО ВИДА *STREPTOCOCCUS INTERMEDIUS*, ВЫЗВАВШЕГО АБСЦЕСС ГОЛОВНОГО МОЗГА, СЕКВЕНИРОВАНИЕМ ПО СЭНГЕРУ ГЕНА 16S РРНК ИЗ ДНК ОБРАЗЦА ГНОЯ

М. А. Гордукова[1] ✉, Ю. В. Дивилина[1], О. В. Мишукова[2], Е. В. Галеева[1], А. П. Продеус[1], М. Л. Филипенко[2]

[1] Клиническая диагностическая лаборатория,
Детская городская клиническая больница № 9 им. Г. Н. Сперанского, Москва

[2] Лаборатория фармакогеномики,
Институт химической биологии и фундаментальной медицины СО РАН, Новосибирск

Представлено описание клинического случая: наблюдали девочку в возрасте 14 лет 11 мес с абсцессом головного мозга, для которого не удалось установить возбудителя стандартными микробиологическими методами. До и в течение периода госпитализации у ребенка отсутствовала лихорадка, а многие антибиотики вызывали аллергические реакции, в связи с чем диагностика и терапия инфекции были затруднены. Пациентке были выполнены три операции. Четырежды производили посев гноя из абсцесса, но ни разу не наблюдали роста культуры. Дважды проводили ПЦР-анализ, но в обоих случаях результаты исследования были отрицательными. Тогда был амплифицирован и секвенирован по Сэнгеру ген 16S рРНК из образца ДНК, экстрагированной из гноя. С помощью программы BLAST была показана высокая гомология (99 %) определенной последовательности с последовательностью гена 16S рРНК бактерии *Streptococcus intermedius* (штамм ChDC B589, KF733728.1), для которой ранее была описана роль в развитии абсцессов головного мозга. Терапия *ex juvantibus* против этого микроорганизма, начатая еще до получения результатов секвенирования, привела к положительной динамике и купированию процесса у ребенка. Таким образом, в отдельных случаях секвенирование может являться практически единственным способом идентификации потенциального возбудителя.

Ключевые слова: абсцесс головного мозга, *Streptococcus intermedius*, 16S рРНК, секвенирование по Сэнгеру, лабораторная диагностика

✉ **Для корреспонденции:** Гордукова Мария Александровна
Шмитовский пр-д, д. 29, г. Москва, 123317; ma.gordukova@dgkb-9.ru

Статья получена: 04.07.2017 **Статья принята к печати:** 04.08.2017

The first step in combating infection is rapid and accurate identification of its causative agents. This aids the choice of adequate antibacterial therapy for treating clinical syndromes, such as sepsis, as well as conditions caused by multiple pathogens, including infections of the upper respiratory tract.

As a rule, laboratories use culture-based methods to identify pathogens and test them for drug susceptibility. This may be time-consuming and ineffective for poorly culturable microorganisms or those whose viability was compromised by previous antibacterial treatment. Current molecular tests are highly sensitive and specific enough to identify a great variety of pathogens directly in clinical samples [1].

The list of pathogens that can be identified using routine tests is limited to the most common bacterial species, which is why culture and molecular genetic tests sometimes come out negative in patients showing signs of infection. As early as the 1990s, an approach was proposed for pathogen identification based on the amplification of the 16S rRNA gene region from a clinical sample or pure bacterial culture, followed by its sequencing and comparison of the obtained nucleotide sequence with those of known pathogens [2].

Because the16S rRNA gene is ubiquitous in bacteria and highly conserved, it is a perfect target for bacterial identification [3]. Its conserved regions are interspersed with hypervariable ones, and the combinations of the two are species-specific. The 16S rRNA gene is amplified using oligonucleotide primers complementary to its conserved regions [4]. The nucleotide sequence of a PCR product is then analyzed and compared to known sequences available in such databases as RiboDB, revealing the identity of a pathogen. So far, dozens of thousands of nucleotide sequences have been described of the 16S rRNA gene characteristic of different bacterial species.

DNA for amplification and sequencing can be obtained either from clinical samples or bacterial isolates, especially when the list of potentially involved pathogens is very long. This method can be applied to poorly culturable bacteria or clinical samples of patients who received antibiotics before testing [5–7]. Some researchers used cerebrospinal fluid, pus, synovial and interstitial fluids for 16S rRNA gene isolation [8]. For example, Xia et al. [9] sequenced the 16S rRNA gene in parallel with doing the standard culture testing to identify infectious agents in patients with pneumonia. A few genera that standard culture techniques failed to detect were identified by sequencing, namely *Prevotella*, *Proteus*, *Aquabacter* and *Sphingomonas*. Both sequencing and standard culture identified *Streptococcus*, *Neisseria*, *Corynebacterium*, *Acinetobacter*, *Staphylococcus*, *Pseudomonas* and *Klebsiella*, but sequencing was more sensitive in the case of *Streptococcus* and *Pseudomonas*. In another work by Daroy et al. [10] sequencing of the 16S rRNA gene revealed the presence of bacterial *Haemophilus influenzae*, *Sphingomonas sp*., *Klebsiella pneumoniae*, *Staphylococcus haemolyticus*, *Morganella morganii*, *Mycobacterium sp*., *Chryseobacterium sp*., *Pseudomonas saccharophila* (*Xanthomonas*) and fungal *Phaeoacremonium inflatipes* in 19 samples of lacrimal fluid of patients with eye infection whose standard cultures came out negative.

In this article we describe a clinical case of brain abscess caused by a pathogen that could be identified only by amplification and sequencing of the 16S rRNA gene.

Case description

A girl aged 14 years 11 months diagnosed with brain abscess was in care in Speransky Children's Clinical Hospital No. 9 (Moscow) from June 6 to July 15, 2016. The diagnosis was delayed and treatment was complicated by the absence of fever before and after hospital admission and by allergic reactions to a wide range of antibiotics

Starting from January 2016, the girl had been complaining of pain in the right ear. On May 25 she was diagnosed with right-sided acute catarrhal otitis media. When the child started to feel nauseous and vomited, an ambulance was called, and the girl was taken to the hospital. She did not have fever. The following day the patient developed nuchal rigidity and ataxia; Kernig's and Brudzinski's signs (in both upper and lower limbs) were positive. A CT scan showed abscess in the right cerebral hemisphere accompanied by perifocal edema.

On June 7 craniotomy was performed, 2 ml of pus were evacuated from the right parietal lobe, a drain was installed, and the patient was prescribed antibacterial treatment. However, in response to vancomycin the patient developed rashes, therefore, the drug was replaced with a combination of meropenem (2 g per day) and linezolid (600 mg every 12 hours). On June 9 the allergic reaction to linezolid was observed: the patient became nauseous, vomited and had difficulty breathing. Linezolid was replaced with amikacin (400 mg twice a day). On June 13 the drain was removed. On June 19 the girl's mother asked to discontinue metropenem because the child had developed allergy to this drug.

On June 21 contrast enhanced CT was performed revealing a recurrence of abscess in the neighboring brain regions. The patient was prescribed cefepime (2 g three times a day) and i. v. metronidazole (0.5 g a day). On the same day the patient had another craniotomy; 20 ml of pus were evacuated. The patient did not have fever. No purulent discharge was visible coming through the drain after the surgery. On June 25 another intervention was performed to evacuate another 12 ml of pus. The body temperature was normal. Vancomycin was added to the regimen; on June 30 the child started to receive cefomax instead of cefepime (2 g three times a day). In total, the patient had 3 surgeries during her stay in the hospital.

None of four pus samples were culture positive. Results of blood chemistry tests including the levels of C-reactive protein and erythrocyte sedimentation rate are shown in the table below.

Twice, on June 14 and 22, brain abscess specimens were tested by PCR for herpes simplex virus types I, II and IV, cytomegalovirus, Epstein—Barr virus, enteroviruses, *Toxoplasma gondii*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Haemophilus influenza*, *Streptococcus pneumoniae*, methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*, methicillin-resistant coagulase-negative strains of *S. aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. glabrata*, *C. krusei* and *Streptococcus spp*. The analysis was carried out on DNA and cDNA. Both times the tests

Levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) of the patient

Date	CRP, mg/l	ESR, mm/h
07.06.2016	**55.8**	–
14.06.2016	3.1	**46**
20.06.2016	2.2	–
27.06.2016	0.9	–
01.07.2016	0.6	19
12.07.2016	0.5	19

Note. CRP reference values 0.1–8.2 mg/l; ESR reference values 0–20 mm/h. Values outside the reference interval are shown in bold.

were negative. The actin-coding gene was used as an internal control; the amplification curve crossed the threshold at Ct = 23.39, which indicated good DNA quality.

A DNA sample was sent to the Laboratory of Pharmacogenomics of the Institute of Chemical Biology and Fundamental Medicine SB RAS to amplify the fragment of the 16S RNA gene. Sequencing yielded a nucleotide stretch with highly variable regions v2–v4 of the gene. BLAST analysis (NCBI, USA) revealed a 99 % homology of the obtained sequence to the sequence of the 16S rRNA gene characteristic of *Streptococcus intermedius* (strain ChDC B589, KF733728.1).

Case discussion

The literature and international clinical guidelines describe the role of a commensal *Streptococcus intermedius* in the development of brain abscess [11]. Also a case is known of an elderly woman with brain abscess whose aspirates were culture negative, and the causative agent was identified only by sequencing of the 16S rRNA gene [12]. *Streptococcus intermedius* and *Streptococcus constellatus* constitute a group, which is sometimes referred to as Streptococcus milleri group, demonstrating a great serologic and hemolitic variety and varying immunogenicity. Perhaps, *S. intermedius* did not trigger an adequate immune response in our patient, resulting in the absence of fever and markers of inflammation (see the Table). As a matter of fact, no laboratory techniques available in our multi-specialty medical facility but Sanger sequencing could have identified the pathogen.

Negative PCR results could probably be explained by the absence of a reference strain in the reagent kit used for the assay. Treatment *ex juvantibus* against *S. Intermedius* was started before sequencing results were available. The patient responded positively and her condition improved, which is consistent with sequencing results. Therefore, the studied clinical case demonstrates that if PCR and Sanger sequencing yield discordant results, Sanger sequencing should be given diagnostic priority as the gold standard molecular technique.

CONCLUSIONS

Amplification and sequencing of the entire 16S rRNA gene or its fragments directly from clinical samples have their own limitations. Samples should be collected from sterile sites. Polymicrobial infections and sampling from nonsterile sites impede accurate interpretation of sequencing data. Besides, even if an amplicon of microbial DNA is obtained from a clinical sample, it does not necessarily indicate that the identified microorganism is the cause of the infection. Sequencing of the 16S rRNA gene is labor intensive and requires good equipment and high qualifications of the lab personnel. But in some cases it remains the only method capable of identifying the pathogen. In the described clinical case sequencing of the 16S rRNA gene allowed us to identify an atypical bacterial strain. Having analyzed the literature, we were able to hypothesize its role in the development of brain abscess and suggest the reasons for the atypical course of the disease.

References

1. Lehmann LE, Hunfeld KP, Emrich T, Haberhausen G, Wissing H, Hoeft A et al. A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples. Med Microbiol Immunol. 2008 Sep; 197 (3): 313–24. DOI: 10.1007/s00430-007-0063-0.
2. Bruce IJ. Nucleic acid amplification mediated microbial identification. Sci Prog. 1993–1994; 77 (Pt 3–4): 183–206.
3. Woese CR. Bacterial evolution. Microbiol Rev. 1987 Jun; 51 (2): 221–71.
4. Relman DA. The search for unrecognized pathogens. Science. 1999 May 21; 284 (5418): 1308–10.
5. Schmidt TM, Relman DA. Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. Methods Enzymol. 1994; 235: 205–22.
6. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. Clin Microbiol Rev. 2001 Jan; 14 (1): 177–207. DOI: 10.1128/CMR.14.1.177-207.2001.
7. Harris KA, Fidler KJ, Hartley JC, Vogt J, Klein NJ, Monsell F et al. Unique case of Helicobacter sp. osteomyelitis in an immunocompetent child diagnosed by broad-range 16S PCR. J Clin Microbiol. 2002 Aug; 40 (8): 3100–3.
8. Harris KA, Hartley JC. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. J Med Microbiol. 2003 Aug; 52 (Pt 8): 685–91. DOI: 10.1099/jmm.0.05213-0.
9. Xia LP, Bian LY, Xu M, Liu Y, Tang AL, Ye WQ. 16S rRNA gene sequencing is a non-culture method of defining the specific bacterial etiology of ventilator-associated pneumonia. Int J Clin Exp Med. 2015 Oct 15; 8 (10): 18560–70.
10. Daroy ML, Lopez JS, Torres BC, Loy MJ, Tuano PM, Matias RR. Identification of unknown ocular pathogens in clinically suspected eye infections using ribosomal RNA gene sequence analysis. Clin Microbiol Infect. 2011 May; 17 (5): 776–9. DOI: 10.1111/j,1469-0691.2010.03369.x.
11. Mishra AK, Fournier PE. The role of Streptococcus intermedius in brain abscess. Eur J Clin Microbiol Infect Dis. 2013 Apr; 32 (4): 477–83. DOI: 10.1007/s10096-012-1782-8.
12. Saito N, Hida A, Koide Y, Ooka T, Ichikawa Y, Shimizu J et al. Culture-negative brain abscess with Streptococcus intermedius infection with diagnosis established by direct nucleotide sequence analysis of the 16s ribosomal RNA gene. Intern Med. 2012; 51 (2): 211–6.

Литература

1. Lehmann LE, Hunfeld KP, Emrich T, Haberhausen G, Wissing H, Hoeft A et al. A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples. Med Microbiol Immunol. 2008 Sep; 197 (3): 313–24. DOI: 10.1007/s00430-007-0063-0.
2. Bruce IJ. Nucleic acid amplification mediated microbial identification. Sci Prog. 1993–1994; 77 (Pt 3–4): 183–206.
3. Woese CR. Bacterial evolution. Microbiol Rev. 1987 Jun; 51 (2): 221–71.
4. Relman DA. The search for unrecognized pathogens. Science. 1999 May 21; 284 (5418): 1308–10.
5. Schmidt TM, Relman DA. Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. Methods Enzymol. 1994; 235: 205–22.

6. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. Clin Microbiol Rev. 2001 Jan; 14 (1): 177–207. DOI: 10.1128/CMR.14.1.177-207.2001.
7. Harris KA, Fidler KJ, Hartley JC, Vogt J, Klein NJ, Monsell F et al. Unique case of Helicobacter sp. osteomyelitis in an immunocompetent child diagnosed by broad-range 16S PCR. J Clin Microbiol. 2002 Aug; 40 (8): 3100–3.
8. Harris KA, Hartley JC. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. J Med Microbiol. 2003 Aug; 52 (Pt 8): 685–91. DOI: 10.1099/jmm.0.05213-0.
9. Xia LP, Bian LY, Xu M, Liu Y, Tang AL, Ye WQ. 16S rRNA gene sequencing is a non-culture method of defining the specific bacterial etiology of ventilator-associated pneumonia. Int J Clin Exp Med. 2015 Oct 15; 8 (10): 18560–70.
10. Daroy ML, Lopez JS, Torres BC, Loy MJ, Tuano PM, Matias RR. Identification of unknown ocular pathogens in clinically suspected eye infections using ribosomal RNA gene sequence analysis. Clin Microbiol Infect. 2011 May; 17 (5): 776–9. DOI: 10.1111/j,1469-0691.2010.03369.x.
11. Mishra AK, Fournier PE. The role of Streptococcus intermedius in brain abscess. Eur J Clin Microbiol Infect Dis. 2013 Apr; 32 (4): 477–83. DOI: 10.1007/s10096-012-1782-8.
12. Saito N, Hida A, Koide Y, Ooka T, Ichikawa Y, Shimizu J et al. Culture-negative brain abscess with Streptococcus intermedius infection with diagnosis established by direct nucleotide sequence analysis of the 16s ribosomal RNA gene. Intern Med. 2012; 51 (2): 211–6.