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Short report

Real-time metagenomics next-generation sequencing for diagnosing polymicrobial meningoencephalitis in Niger: A case report

Abdourahamane Yacouba [1,2,*], Moumouni Garba [1,2], Marou Soumana Boubacar [1,3], Sahada Moussa Saley [1,3], Samaila Aboubacar [1,2], Abdoulaye Ousmane [4], Ahmed Olowo-Okere [5], Souleymane Brah [1,2], Mamane Daou [1,3], Mahamadou Doutchi [6], Eric Adehossi [1,7], Saidou Mamadou [1,2] and Madjid Morsli [8]

1. *Faculté des Sciences de la Santé, Université Abdou Moumouni, PB 10896, Niamey, Niger*
2. *Hôpital National Amirou Boubacar Diallo, PB 1364 Niamey, Niger*
3. *Hôpital National de Niamey, PB 238 Niamey, Niger*
4. *Faculté des Sciences de la Santé, Université Dan Dicko Dan Kolodo, PB 465 Maradi, Niger*
5. *Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Abuja, PB 2346 Abuja, Nigeria*
6. *Faculté des Sciences de la Santé, Université André Salifou, PB 656 Zinder, Niger;*
7. *Hôpital Général de Référence, PB 12674 Niamey, Niger*
8. *VBIC, INSERM U1047, Univ Montpellier, Service de Microbiologie et Hygiène Hospitalière, CHU Nîmes, Cedex 9, 30029 Nîmes, France*

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ABSTRACT

Real-time metagenomics next-generation sequencing (RT-mNGS) has emerged as a high-throughput technique for directly identifying pathogen genomes from clinical samples. This study aimed to document a case of nonroutinely diagnosed meningoencephalitis using RT-mNGS in the Niger Republic. **Case presentation** The patient was a 12-year-old African Nigerien female who reported fever for two weeks before she visited our hospital, as her fever had worsened with focal neurologic deficits. All other physical examinations yielded no notable findings or abnormalities. A brain abscess was first presumed in this patient and empirical treatment with ceftriaxone and metronidazole was initiated before diagnosis. Conventional bacteriological tests yielded negative results and the lesions seen on brain-computed tomography images are not specific for brain abscesses. Using real-time metagenomic next-generation sequencing (RT-mNGS), the pathogen's genomes were detected after a one-hour sequencing run, directly from leftover cerebrospinal fluid. WU polyomavirus strain W33 (GenBank accession no: GU296367.1) was identified in the patient's sample. *Haemophilus influenzae*-specific reads (667 reads) were also detected, which explains the possible bacteria–DNA virus coinfection in this case. Furthermore, *Achromobacter xylosoxidans* (599 reads) was detected in this patient. Based on these findings, we classified this case as polymicrobial meningoencephalitis. **Conclusion** This case report highlights the significance of RT-mNGS in diagnosing non-routinely detectable pathogens. RT-mNGS can rapidly and accurately characterize meningoencephalitis pathogen and might contribute to a reduction in mortality. However, the issue of sample contamination should always be addressed with high priority.

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* *Corresponding author:* Abdourahamane Yacouba

E-mail address: abdourahamane.yacouba@uam-cfrmt.ne

Introduction

Community-acquired meningoencephalitis (CAME) is a life-threatening condition characterized by acute central nervous system (CNS) inflammation caused by a microbial infection in 30–50% of cases [1]. In most cases of CNS infections, viral and bacterial pathogens are the primary causative agents, while CNS infections caused by fungi and parasites have also been reported in the literature, predominantly among immune-compromised patients [2–5]. The clinical manifestation of CAME depends on the causative pathogen and the inflammation level, including fever, headache, neck stiffness, confusion, seizures, nausea, vomiting, photo/phonophobia, and loss of consciousness and coma in specific situations [6].

The current diagnostic approach for CAME includes comprehensive physical examination, imaging techniques such as a CT scan or magnetic resonance imaging (MRI), and cerebrospinal fluid (CSF) analysis [7]. The gold standard for the routine diagnosis of CAME continues to be CSF culture, despite its limitations, with a failure rate ranging from 10% to 56% [8]. Viruses remained the primary cause of encephalitis across age groups, yet viral culture may not be appropriate as a routine procedure. Additionally, CSF culture for bacterial or fungal infections may take several days, delaying the final diagnosis, patient management, and treatment.

To enhance diagnostic accuracy, multiplex reverse transcription polymerase chain reactions are employed, targeting a restricted panel of pathogens commonly associated with CNS diseases [9]. The main limitation of the multiplex tests is the inability to detect all potential emergent pathogens and that a negative test result does not rule out the possibility of infection.

Real-time metagenomics next-generation sequencing (RT-mNGS) has emerged as a high-throughput technique for directly identifying pathogen genomes from CSF samples in real-time [10–13]. This cutting-edge approach eliminates the need for the prior amplification or targeting of specific genetic markers, rendering RT-mNGS a universal method capable of diagnosing both monomicrobial and polymicrobial infections directly from clinical samples, as well as an in silico prediction of antibiotic susceptibility profiles and genotypes associated with these pathogens [14]. Herein, we report the first case of polymicrobial CAME in a pediatric patient in the Niger Republic, diagnosed via RT-mNGS applied directly to a CSF sample.

Case Presentation

Baselines characteristics

A 12-year-old African Nigerien female patient was admitted to the pediatric emergency unit of Amirou Boubacar Diallo National Hospital, Niamey, the Niger Republic, with complaints of fever, intense and progressive headache, and focal neurologic deficits. The patient was living with her family in Niamey, and no chronic disease, or significant past medical history, or specific medication, or medical advice had been reported. At admission, the patient was awake, confused, and unresponsive. Her Glasgow Coma Scale (GCS) score was 12, and her vital signs were as follows: temperature—38.2 °C, blood pressure—180/120 mmHg, pulse rate—121 beats/min, and respiratory rate—21 breaths/min with oxygen saturation (SpO_2) of 96% in ambient/room air. A neurological examination revealed right hemiplegia without neck stiffness. Based on the scoring system proposed by the Medical Research Council (MRC), the patient had muscle power of 0/5 and 0/5 in the right upper and lower extremities, respectively. Deep tendon reflexes were symmetrical, and flexor plantar reflexes were present. All other physical examinations yielded no notable findings or abnormalities. A CSF analysis after lumbar puncture showed a blood cell count of 46/µL (97% lymphocytes), a normal glucose level, and a protein level of 41 mg/dL. Blood analysis was significant for leukocytosis, profound hyponatremia (118 mmol/L), and elevated levels of creatinine and urea; the C-reactive protein level (CRP) was increased (9.50 mg/dL), and direct blood cultures were negative. A brain abscess was first presumed in this patient. The brain-computed tomography (CT) scan without contrast showed bright cortical sulci with asymmetric white matter lesions visible as areas of low attenuation **(Figure S1)**. Unfortunately, these lesions seen on CT images are not specific for brain abscesses. The National Institute of Health Stroke Scale (NIHSS) score was 7 points. Empirical intravenous ceftriaxone and metronidazole were initiated pending diagnostic confirmation.

Against medical advice and at the request of her parents, the patient left the hospital after 10 days of hospitalization due to financial constraints.

Unfortunately, the patient died two days later, although all routine microbiological investigations, including molecular assays and CSF culture, were negative.

Real-time metagenomics next-generation sequencing

A few days later, metagenomic-based real-time sequencing was applied directly to leftover CSF samples recovered after routine assays to detect all potential pathogen genomes nonroutinely targeted by RT–PCR assays. The rationale behind the choice of this technique is that metagenomics enables the genome of microbial pathogens to be detected directly from the LCS, without the need for specific targets.

– **DNA/RNA extraction**

Total DNA/RNA was extracted from 50 µL of leftover CSF using EZ1 Virus Mini Kit v2.0 (Qiagen, Courtaboeuf, France) after treatment with proteinase K for 20 min at 56 °C; it was then eluted in a 50 µL final volume. cDNA synthesis was performed following the TaqMan (Applied Biosystem, Waltham, MA, USA) protocol; a 50 µL final volume containing 50 µM MgCl2, 10× buffer, 10 mM dNTPs, 1 µM random primers, 5 units of Superscript III, and 5 µL RNase inhibitor was prepared. Then, the volume was adjusted to 50 µL with the extracted RNA and incubated in a thermal cycler (Applied Biosystems) following a standard protocol—10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C to inactivate the RNase inhibitor. Double-stranded cDNA synthesis was performed in a 30 µL volume containing 10× Neb buffer 2, 10 µM dNTPs, nuclease-free water, 3 units of DNA Polymerase I, large (Klenow) fragment (BioLabs), and 20 µL cDNA. The double-stranded synthesis was conducted at a duration of 2 h at 37 °C; it was then purified using 0.5 Ratio Agencourt® AMPure beads and eluted in 20 µL of 1x sterile Tris-EDTA buffer.

– **Oxford Nanopore library preparation**

A 48 µL mix of the extracted DNA/cDNA was used for Oxford Nanopore library preparation following an in-house improved protocol and then indexed and purified in a final 75 µL volume, as previously described [7,8]. The final library was sequenced in an 8 h single-end long-read run using an Oxford Nanopore MinION (Oxford Nanopore technologies, Oxford, UK).

– **Illumina Nextera-XT library preparation**

In parallel, 1 ng DNA/RNA was used for paired-end Illumina Nextera-XT library preparation as previously described [8] to confirm the MinION results. Briefly, 5 µL of standardized DNA was fragmented in a mix containing 5 µL of Amplicon Tagment Mix in the presence of Tagment DNA Buffer (Nextera XT Library Prep Kit, Illumina) for 5 min at 55 °C in an ABI 2720 GeneAmp PCR System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in a 20 µL volume. Then, 5 µL of Neutralize Tagment Buffer was added before centrifugation for 1 min at 2800× *g* and 5 min incubation at room temperature; they were then amplified in a 50 µL volume in the presence of Nextera® XT Index Kit V2, San Diego, CA, USA). The first purification was performed using Agencourt Ampure XP beads (Beckman Coulter, Villepinte, France) in a 0.8 ratio of beads, followed by two washes with 80% alcohol and elution in 52.5 µL of RSB buffer. The library concentration was measured in an Agilent 2100 Bioanalyzer (Thermo Fisher Scientific, Santa Clara, CA 95051, United States) and then diluted in RSB buffer in the presence of a 10 µL volume of Phix (50 pM). Finally, 50 pM of the diluted libraries was denatured and sequenced on the iSeq 100 sequencer (Illumina, San Diego, CA, USA) in a single 17.5 h run, providing 2 × 150-bp long reads.

– **Metagenomic data analysis and pathogen genome detection**

Real-time analysis of the MinION-generated sequences was performed using the recommended EPI2ME Oxford Nanopore online software (version 2019.11.11-2920621). In parallel, total mNGS data were analyzed by Kraken 2 on Galaxy online software (https://usegalaxy.eu/, accessed on 16 February 2023) and visualized using Pavian online software (https://fbreitwieser.shinyapps.io/pavian/, accessed on 16 February 2023) (Figure 1). Total MinION and Illumina reads were concatenated and assembled using the Spades assembler (version: 3.15.4), blasted against the NCBI GenBank database, and mapped to the hit-BLAST reference genomes using the CLC genomic workbench (Qiagen) (version: 21.1.1). Phylogenetic analysis based on the whole-genome sequencer was used to describe the evolutionary relationships between species. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura

2-parameter model, and the evolutionary analyses were conducted in MEGA7 software (version: 7.0.26). The initial tree for the heuristic search was obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with a superior log-likelihood value.

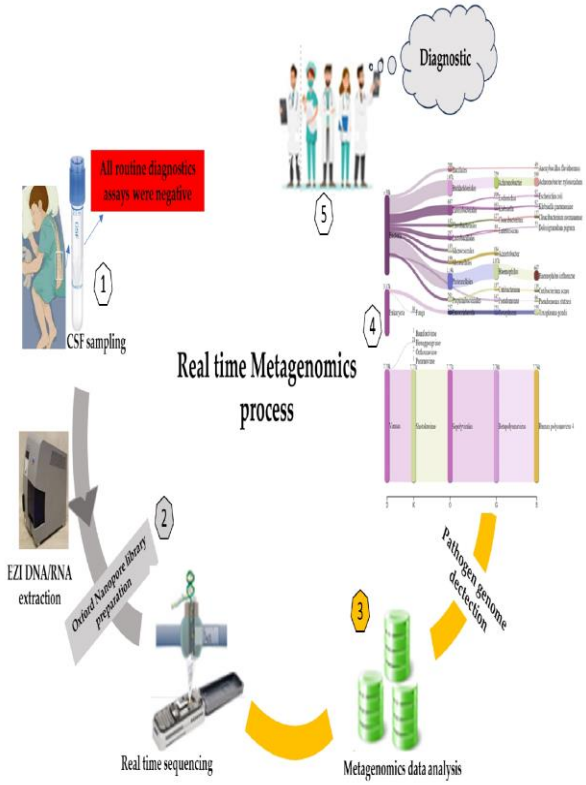

Figure 1. Real-time metagenomics procedure. 1. CSF sampling was done at the reception of the patients at the emergency department. All routine diagnostic assays were negative. 2. DNA/RNA extraction using EZ1 Virus Mini Kit v2.0 (Qiagen). Oxford Nanopore MinION Library preparation and sequencing. 3. MinION data analysis in real-time by EPI2ME, then with Kraken and Pavian online software. 4. The final genome sequences were recovered using a CLC Genomic workbench (Qiagen). 5. Results sent to physician.

Results and Discussion

Real-time analysis of MinION data detected 132 WU Polyomavirus-specific reads after a one-hour run. The BLAST nucleotide of the FASTA sequences after the assembly of both Illumina and Nanopore reads against the NCBI GenBank database identified WU polyomavirus strain W33 (GenBank accession no: GU296367.1), isolated from a pediatric Australian patient [9]. The BLAST sequence alignment exhibited 100% coverage and 99.98% identity. A mapping of the total reads against the hit-BLAST sequence (GenBank accession no: GU296367.1) using CLC software showed that 11,632/1,048,634 (1.12%) of the reads were matched with the reference genome, generating 5228 bp corresponding to 100% polyomavirus genome coverage with 165 mean depths. Phylogenetic analysis based on the whole-genome sequence of the identified Polyomavirus strain (BANPedHNABD), the six hit-BLAST WU Polyomaviruses, and other representative polyomavirus genomes recovered from the GenBank database showed that the identified Polyomavirus sequence clustered with other WU Polyomavirus isolates, mainly with WU polyomavirus isolate NP360 (GenBank accession no: GU296402) at 82% sequence similarity **(Figure 2)**; this isolate belongs to the human polyomavirus 4 family, isolated from patients with acute respiratory infection [9]. The pathogenicity of WU polyomavirus and its association with CSN prognosis remains unclear. Despite its previous detection in CSF samples [15,16], no cases of WU polyomavirus meningoencephalitis have been reported at present. It is far more likely that the WU polyomavirus is an incidental finding related to virus reactivation or chronic shedding rather than being the etiologic cause of the patient's meningitis [17]. WU polyomavirus is a persistent virus that is reactivated by an inflammatory process or a predisposing or aggravating factor [14]. Primary infection with the WU polyomavirus probably occurs early in childhood, as suggested by the seroprevalence in young people [18]. JC and BK polyomaviruses are the most targeted polyomaviruses in routine diagnoses, commonly associated with respiratory and CNS diseases [19,20], whereas the WU polyomavirus is completely neglected in routine diagnoses.

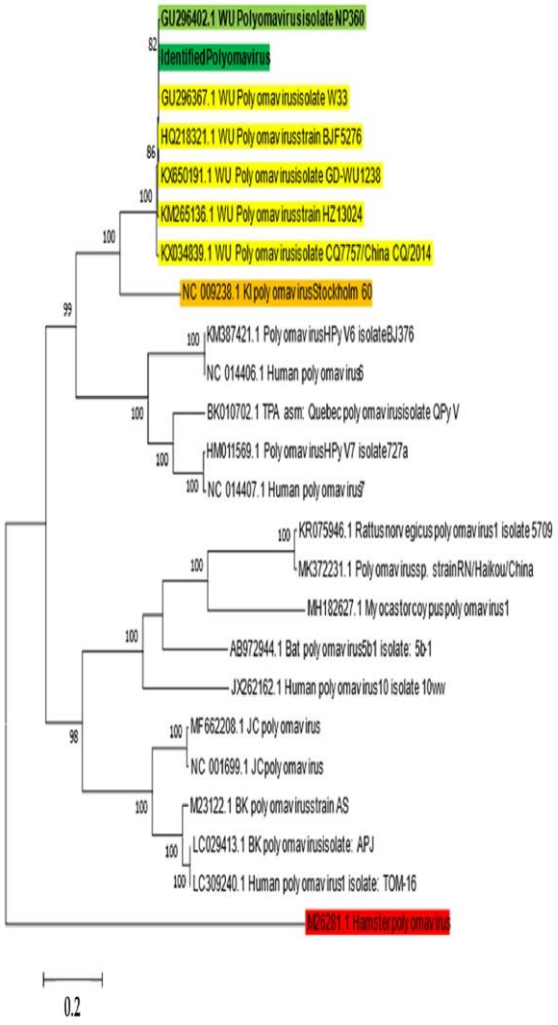

Figure 2. Phylogenetic analysis based on the polyomavirus whole genome sequence of the identified polyomavirus, hit-BLAST sequences, and other representative polyomaviruses recovered from the GenBank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model, and the evolutionary analyses were conducted in MEGA7 software (version: 7.0.26). Light green color: the most closely related Polyomavirus. Dark green color: Polyomavirus isolate from this study. Yellow color: other WU polyomaviruses isolates. Orange color: KI Polyomavirus. Red color: Hamster polyomavirus, used as outgroup.

Haemophilus influenzae-specific reads (667 reads) were detected, which explains the possible coinfection of bacteria–DNA virus in this case. After a total read assembly, Blastn identified *H. influenzae* strain PittGG (GenBank accession no CP000672), belonging to the nontypable *H. influenzae* genotype via multilocus sequence typing analysis, as *H. influenzae* CNS infection had been reported in both young and elderly patients [8]. Since the introduction of the *Haemophilus influenzae* type b vaccine, nontypeable strains of *H. influenzae* have emerged as a significant cause of invasive infections, including meningitis and encephalitis, in indigenous children [21,22]. However, this may also have resulted from the contamination of the specimens. Approximately 44% of children are asymptomatically colonized with nasopharynx nontypable *H. influenzae* in the first two years of life [23].

Furthermore, *Achromobacter xylosoxidans* (599 reads) was detected in this patient. *Achromobacter xylosoxidans* is a gram-negative bacterium that was first isolated from human ear discharge [24]. Since then, *Achromobacter xylosoxidans* has been documented as an opportunistic pathogen in numerous health care-associated infections, including meningitis [25,26]. However, *Achromobacter xylosoxidans* seems to be a weakly virulent pathogen in immunocompetent patients and may even contaminate hospital consumables such as disinfectants (chlorhexidine) and medical devices used for cerebrospinal fluid collection [27].

In addition, the 255 reads specific to *Toxoplasma gondii* that were identified in the sequenced CSF could be the direct cause of CAME, as previously documented in CNS toxoplasmosis in young patients [28] **(Figure 3)**, which could facilitate other bacterial and viral infections. *Toxoplasma gondii* is one of the most common opportunistic parasites of the CNS in HIV-infected patients [29]. Regarding our patient, HIV serology was negative. In immunocompetent patients, *Toxoplasma gondii* is usually asymptomatic [29]. Although it is rare, *Toxoplasma gondii* CNS infection can present as meningoencephalitis during primary infection in an immunocompetent patient [30]. The intense and progressive headache associated with focal neurologic deficits without a clear etiology, in the context of encephalitis, is highly suggestive of toxoplasmosis [29].

Based on our findings, we classified this case as polymicrobial meningoencephalitis, including viral, bacterial, and protozoan infections. Polymicrobial meningoencephalitis is a rare entity in immunocompetent patients, especially in regions with limited healthcare access. Unlike brain abscess, meningoencephalitis is usually presumed to be of a monomicrobial etiology. This condition could lead

to misdiagnosis and mistreatment of meningoencephalitis patients.

Limitations. Despite the detection of multiple microbial genomes potentially involved in CNS prognosis, the direct cause of the disease remains uncertain. The presence of similar protozoan, fungal, bacterial, and parasitic reads in this sample, other than those, may indicate a common presence of contamination in metagenomics. Moreover, possible contamination by the skin microbiota during lumbar puncture remains a limitation for the final interpretation of the mNGS results.

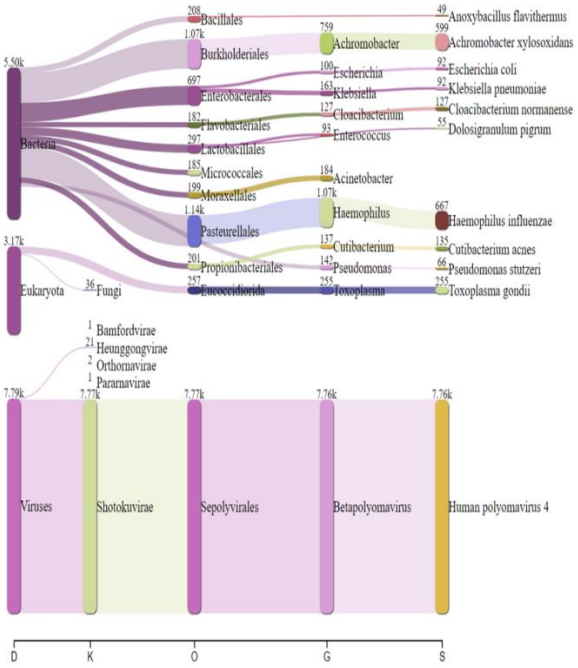

Figure 3. Microorganisms detected in cerebrospinal fluid using metagenomic direct, MinION. Data were visualized in real-time using Pavian (http://github.com/fbreitwieser/pavian, accessed on 16 February 2023).

Conclusions

Polymicrobial meningoencephalitis is a rare entity in immunocompetent patients, especially in the antibiotic era. This case report highlights the utility of RT-mNGS in diagnosis of non-routinely documented pathogens, as well as their genome and genotype in reduced time.

1. Using RT-mNGS, a pathogen genome could be directly detected from a CSF sample, with no specific target.
2. Faced to the high prevalence of routinely undocumented CSFs (≈90%) [8], RT-mNGS provided an opportunity for the early documentation of CNS infection in pediatric populations in endemic countries, as several challenging cases may escape current routine investigations.
3. RT-mNGS opens a new perspective for clinical microbiology laboratories to implement this strategy as an alternative emergency diagnosis of life-threatening CNS infections, facilitating timely clinical decision-making.

Future directions include the use of RT-mNGS to predict pathogen virulence and antimicrobial resistance, enabling prompt and effective treatment.

Supplementary Materials

Figure S1: Brain computed tomography (CT) scan without contrast showing bright cortical sulci with asymmetric white matter lesions visible as areas of low attenuation. (**A**, **B**) CT axial image of the brain showing the lateral ventricles (**A**); (**C**) CT coronal image.

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Author Contributions

A.Y. contributed to the experimental design, routine analyses, data analysis, data interpretation and writing of the first draft. M.G. contributed to clinical diagnosis and CSF sampling. S.M.S. and S.A. collected samples and collected clinical information. M.B.S. performed routine analyses. A.O., A.O.-O., S.B., M.Do., M.Da., E.A. and S.M. contributed to critically reviewing the manuscript and data interpretation and coordinated and directed the work. M.M. contributed to the experimental design, data analysis, bioinformatics data analysis, data interpretation, and writing of the original draft of the manuscript. A.Y. and M.M. ensured the critical review and final validation of the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

All procedures were approved by the Research Ethics Committee of Amirou Boubacar

Diallo National Hospital (HNABD/2022/012, approved on 11 January 2022). Written informed consent was obtained from the patient's guardian (her father) for publication of this case report and any accompanying images.

Competing interests

The authors declare no conflicts of interest.

Availability of data and materials

Real-time metagenomic next-generation sequencing data for this study are available from GenBank under GenBank accession number OP852794.1.

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