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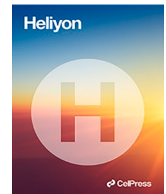

Case report

Droplet digital PCR aids in the diagnosis of children with fever of unknown origin ——A typical case report

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ABSTRACT

Many clinical conditions can cause fever of unknown origin (FUO) in children, but the etiological diagnosis remains challenging despite the variety of inspection methods available at present. This study aims to investigate the effectiveness of droplet digital polymerase chain reaction (ddPCR) in identifying pathogens in children with FUO as a novel application. A 7-month-old boy failed to obtain etiology evidence for his disease through various tests. After collecting peripheral blood for ddPCR analysis, *Staphylococcus aureus* and *Escherichia coli* were detected, and Sanger sequencing confirmed the pathogens. During the disease, the child developed septic arthritis and osteomyelitis in the femur. Despite the patient's fever being removed, his limb activity improving, and inflammatory biomarkers decreasing, avascular necrosis of the femoral head remained after targeted antibiotic treatment and surgery. If the patient had undergone ddPCR analysis at an early stage, it may be possible to avoid sequelae. ddPCR helps identify pathogens in the diagnosis of children with FUO and could be a promising complementary tool.

1. Introduction

Fever is a common symptom of many underlying diseases in children, including infectious diseases, tumor diseases, rheumatism conditions, and others. Most patients with fever can be quickly diagnosed through medical history, physical examination, and basic laboratory tests. However, some children suffer from long-term fevers without a clear source, known as fever of unknown origin (FUO). A FUO in adults was first described in 1961 as a fever persisting for more than three weeks without a clear source after an investigation of one week [1]. Pediatric FUO is not defined in a standard way, but a fever that lasts for 7 days to 3 weeks is generally considered acceptable for diagnosis [2,3]. Various international guidelines suggest a comprehensive laboratory examination for pediatric patients

Abbreviations: FUO, fever of unknown origin; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; qRT-PCR, quantitative real-time polymerase chain reaction; mNGS, metagenomic next-generation sequencing; ddPCR, droplet digital polymerase chain reaction; cfDNA, cell-free DNA; *E.coli*, *Escherichia coli*; *S.aureus*, *Staphylococcus aureus*; MRI, magnetic resonance imaging.

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with FUO, such as white blood cells, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), procalcitonin, blood culture, chest radiograph [4]. Nevertheless, there are ongoing difficulties in the diagnosis of numerous cases. A large portion of pediatric FUO causes (40 %–50 %) can be traced back to infections, emphasizing the need for thorough pathogen screening and the use of blood pathogen nucleic acid detection as advised by some guidelines [4]. However, despite their widespread use in clinical settings, quantitative real-time polymerase chain reaction (qRT-PCR) and metagenomic next-generation sequencing (mNGS) may miss the pathogens due to the low levels of nucleic acid in samples [5].

Compared to qRT-PCR and mNGS, droplet digital polymerase chain reaction (ddPCR) is a one-step amplification technique that has been proven to provide increased sensitivity in detecting nucleic acids [5,6]. Recently, ddPCR has shown promising potential in the fight against infections, but its application in FUO has not yet been adequately explored [7]. The infectious causes of FUO are complicated and varied, with significant differences among populations of different ages, regions, and immunologic status. Here, we selected a ddPCR detection panel for 12 common bacteria, 4 common fungi, and 8 antimicrobial-resistance genes according to the epidemiological survey of infectious agents responsible for pediatric FUO based on the global and Chinese populations [8–12]. Our study aimed to investigate the value of this ddPCR panel as a pathogen screening tool.

We applied this detection panel to the diagnosis and treatment of a 7-month-old child with FUO and reported this case which has followed from etiologic clarification and targeted treatment to clinical recovery using ddPCR analysis.

2. Materials and methods

2.1. Sample collection

Five milliliters of the child's peripheral blood were taken immediately on the day of admission. The blood was centrifuged at 1600*g and 22 °C for 15 minutes to separate the plasma. Cell-free DNA (cfDNA) was extracted from the plasma using a Magnetic Serum/Plasma DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) and an Auto-Pure 10B nucleic acid purification system (Allsheng Instruments Co., Ltd., Hangzhou, China) [13]. The cfDNA was then eluted in 60 μL of 10 mM Tris-EDTA buffer (a solvent that can dissolve DNA and protect it from degradation) and used for ddPCR analysis on the same day.

2.2. Droplet digital PCR analysis

A total of 12 common bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium, Streptococcus pneumoniae*, *Staphylococcus hemolytic*, *Staphylococcus hominis, Staphylococcus capitis*, and *Staphylococcus epidermidis*), 4 common fungi (*Candida albicans*, *Candida glabrata, Candida parapsilosis,* and *Candida tropicalis*), and 8 antimicrobial-resistance genes (KPC, OXA-48, Imipenem, NDM, MecA, Van A (B), Van M, and Mcr-1) were included in this ddPCR assay. ddPCR analysis was performed using a 5-fluorescence-channel ddPCR system (Pilot Gene Technology Co., Ltd., Hangzhou, China). For each test group containing 4 detection targets, the ddPCR master mix had a final volume of 15 μL, which included 10 μL ddPCR premix (with primers and probes) and 5 μL of sample DNA. Amplification of nucleic acid was performed as follows: 95 °C for 5min, followed by 40 cycles at 95 °C for 15s and 60 °C for 60s. The droplets were loaded into an iScanner to measure the fluorescence signals of 5 different droplets (FAM, VIC, ROX, CY5, and A425) (Pilot Gene Technology Co., Ltd., Hangzhou, China). The minimum detection limit is 0.7 copies/ul [6]. The entire process took 3.5 hours.

2.3. Verification of the pathogens by Sanger sequencing

The pathogens detected by ddPCR were further verified by Sanger sequencing. First, we designed specific primers for the bacterial species and performed nested PCR (Table 1). Nested PCR was performed under the following conditions: Round 1: 95 °C for 10min, 35 cycles of 95 °C for 15s, 56 °C for 30s, and 72 °C for 30s, then 72 °C for 5min. Round 2: 95 °C for 10min, 35 cycles of 95 °C for 15s, 57 °C for 30s, and 72 °C for 30s, then 72 °C for 5min. Subsequent Sanger sequencing was performed by Tsingke Biotech Co., Ltd. (Beijing, China). The sequences were compared using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov), and the species with the highest sequence similarity were considered to be the causative pathogens.

2.4. Ethical

This study was approved by the Ethics Committee of Children's Hospital, Zhejiang University School of Medicine (2021-IRB-191).

Table 1
Nested PCR primers for *Staphylococcus aureus* and *Escherichia coli*.

Species	Nested outer PCR primers (3'→5′)	Nested inner PCR primers (3'→5′)
S. aureus	F: TGATTATAAAAATATCGTTTTGGCTGG	F: TTGGCTGGAAAATATAACTCTCGTATG
	R: TTCTTCACGACTAAATAAACGCTCA	R: CTGAAATCTCATTACGTTGCATCG
E. coli	F: GTTAAAAATCCCGGCCAGA	F: ATCAGAATCAAGCAGGCCAGT
	R: GCCGCGGTAATGGTATTAAA	R: ATACGGTGTGCCTTTTGCC

S. aureus, Staphylococcus aureus; *E. coli, Escherichia coli.*

Informed consent for participation and publication of all images, clinical data, and other data was obtained from his parents.

3. Results

3.1. Case presentation

A 7-month-old Chinese boy (70 cm tall, 10 kg weight) who had a fever for 12 days was admitted to our hospital on October 2, 2021. He initially showed persistent fever and high white blood cell count (17.9*10ˆ9/L) and CRP (121.5mg/L) in his blood (Sept. 20, 2021). According to the medical record, his daily maximum body temperature reached over 38.5 °C. Peripheral blood culture was performed twice at the local hospital, but no bacterial growth was observed. He received moxalactam (20mg/kg/dose, twice daily, intravenous injection, day 1 ~ day 6 of onset, Sept. 20, 2021, ~ Sept. 25, 2021) and piperazine-tazobactam (50mg/kg/dose, twice a day, intravenous injection, day 8 ~ day 10 of onset, Sept.27, 2021, ~ Sept.30, 2021) consecutively. Unfortunately, his symptoms didn't improve and his blood CRP level was still high (114.5mg/L, Sept.27, 2021). During the illness, he also had mild diarrhea, no vomiting, no convulsions, no cough, no rash or other symptoms. In the past, he was healthy and had no history of communicable diseases, trauma, surgery, blood transfusion, or food or drug allergies. He was born in Shangrao, China, and is a member of the Han nationality. He is the second child in his family, to be born naturally, with a birth weight of 2.7kg. After birth, he was fed mixed breast milk and cow powdered milk, and in the 6th month, he was given solid food and grew and developed like as other children. He was vaccinated as prescribed (Bacille Calmette-Guérin vaccine, *Neisseria meningitidis* vaccine, 3 doses of hepatitis B vaccine, 3 doses of polio vaccine, and 4 doses of pertussis-diphtheria-tetanus vaccines were administered). The parents, grandparents, and a 5-year-old brother are all healthy. On admission, he had a body temperature of 38.7 °C, respirations of 40 times/min, pulses of 146 beats/min, and blood pressure of 110/74 mmHg. Physical examination revealed that the anterior fontanel was flat and soft, bilateral lung sounds didn't reach rales, the heart had a steady rhythm without murmurs, and the abdomen was soft and without masses. The neurological examination showed no pathological signs, and the limb movements were normal. There were no rashes on the whole body.

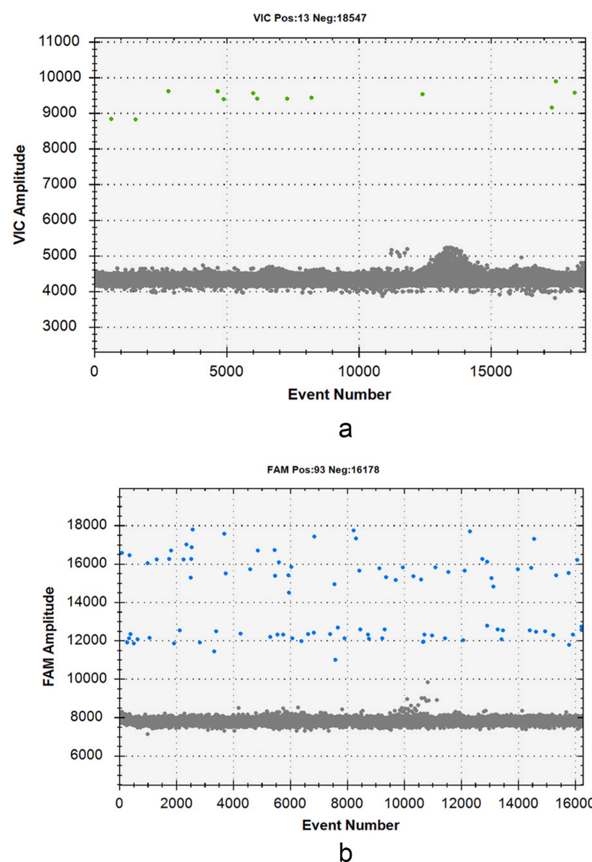


Fig. 1. The detection of DNA of *Escherichia coli* and *Staphylococcus aureus* in the blood by ddPCR analysis. The black dots represent negative controls, while the green and blue dots represent *Escherichia coli* and *Staphylococcus aureus*, respectively. Fig. 1A represents the detection of specific signals in the VIC fluorescence channel, indicating the discovery of *Escherichia coli*. Fig. 1B represents the detection of specific signals in the FAM fluorescence channel, indicating the discovery of *Staphylococcus aureus* in the patient's blood. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Identification and confirmation of pathogens

Peripheral blood ddPCR analysis, synchronized blood culture, and mNGS analysis were conducted on the day of admission (day 12 of onset, Oct. 2, 2021). Two bacterial species (*Escherichia coli* (*E.coli*) (115 copies/ml) and *Staphylococcus aureus* (*S.aureus*) (1115 copies/ml)) were found on ddPCR analysis 5 hours later, but no antimicrobial-resistance genes (Fig. 1). On the second day, we immediately switched to ceftriaxone (100mg/kg/dose, once daily, intravenous injection, starting from Oct. 3, 2021) in combination with linezolid (10mg/kg/dose, every 8 hours, intravenous injection, starting from Oct. 3, 2021). Subsequent Sanger sequencing confirmed the presence of these two species. Synchronized blood culture and mNGS analysis didn't detect any pathogen. The patient's body temperature normalized two days after treatment adjustment, but his right lower extremity presented poor movement on day 17.

3.3. Evaluation of infectious locations

To evaluate the focus of the infection, this patient underwent cerebrospinal fluid analysis, magnetic resonance imaging (MRI) of the head, computed tomography of the chest, abdominal ultrasound, and echocardiography, all of which were negative. However, the X-ray of his hip (Oct. 7, 2021) showed acute inflammatory changes in his right hip joint and femur, suggesting that he may have developed septic arthritis in combination with femoral osteomyelitis (Fig. 2).

3.4. Multimodality therapy

On the 18th day of the illness (Oct. 8, 2021), the orthopedic team performed decompression and reduction surgery on the patient's right hip joint. During the operation, increased pressure was found in the joint capsule, and the joint cavity was filled with pus and covered with a large amount of white purulent moss. The femoral head and metaphysis were corroded. Bacterial culture, ddPCR analysis, and mNGS analysis of the pus were negative. The pathologic findings were consistent with septic arthritis and ruled out tumors and other diseases affecting the hip joint (Fig. 3). The infectious disease team provided continuous antimicrobial therapy and the pediatric rehabilitation team assisted him with ambulation.

3.5. Dynamic changes in inflammatory biomarkers and organ function

To better evaluate the effectiveness of the therapy, we regularly monitor the inflammatory biomarkers and the organ functions in this patient (Fig. 4). The CRP level fell rapidly to below 100mg/L after the targeted antimicrobial treatment for 4 days (Oct. 8, 2021), and reached normal values on the 9th day (Oct. 12, 2021). Other inflammatory biomarkers such as ESR and procalcitonin also decreased rapidly. The organ dysfunction (liver function) caused by the severe infection gradually returned to normal.

3.6. Follow-up

The child was discharged on Oct. 27, 2021. After discharge, the patient continued oral antimicrobial therapy (cefdinir (3mg/kg/dose, 3 times daily) and linezolid (10mg/kg/dose, 3 times daily)) for 4 weeks and rehabilitation therapy until now. Throughout the process, the patient did not experience any adverse events caused by medication or non-medication treatment. From one month (Nov. 21, 2021) to one year (Oct. 31, 2022) after surgery, a series of X-rays of the hip showed that the signs of inflammation gradually subsided (soft tissue swelling), no other signs of persistent infection (such as bone marrow edema), but the femoral head continued to shrink until it disappeared. MRI of the hip (Feb. 27, 2022) three months after surgery showed no signs of chronic infection but did show bone necrosis. The child was confirmed to eventually develop avascular necrosis of the femoral head (Fig. 5, Fig. 6).

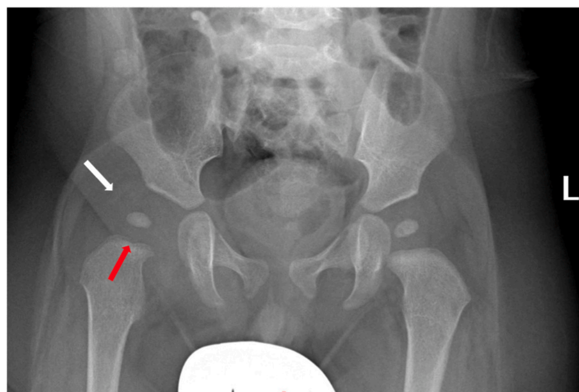


Fig. 2. The patient's hip X-ray (Oct. 7, 2021) shows swelling of soft tissue (white arrow marking), destruction of the right proximal femoral epiphysis (red arrow marking), and pathologic hip joint dislocation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

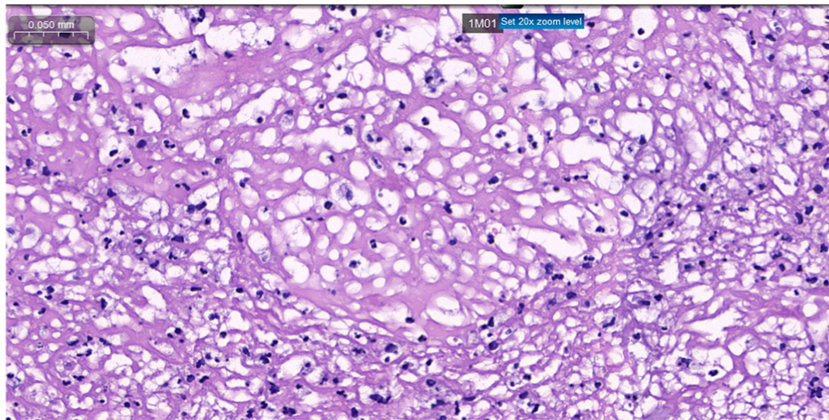


Fig. 3. The pathological report of the patient's hip joint biopsy specimen shows abundant inflammatory necrotic exudate and inflammatory granulation tissue, accompanied by infiltration of lymphocytes, plasma cells, neutrophils, and histiocytes.

4. Discussion

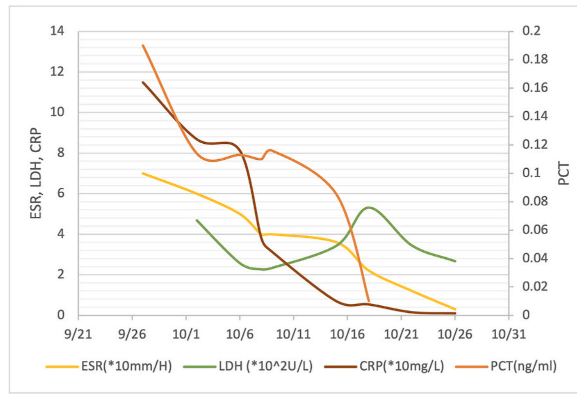
In pediatric FUO, infectious diseases account for the majority, while bone and joint infections are not uncommon. A systematic review of 18 studies with 1638 cases found that bone and joint infections are common causes of children with FUO in developed countries [3]. This patient meets the pediatric diagnostic criteria for FUO according to the latest published guideline (a temperature of more than 38.0 °C lasting longer than at least 8 days without a clear cause) [14].

The incidence rate of septic arthritis in children is estimated at 4–37/100,000, and children under 2 years of age are most commonly affected [15]. The hip joints are most commonly affected, in first or second place [15–17]. Pathogens were identified by blood or synovial fluid culture in only about 1/4–1/2 of patients, while less than 10 % of pathogens were diagnosed by blood culture [15,18]. The most common pathogen is *S. aureus* [18,19]. Infants with septic hips are prone to various complications and sequelae, such as osteomyelitis, avascular necrosis of the femoral head, leg length discrepancy, subluxation, and dislocation [20]. First, symptoms are often absent or atypical in infants, leading to a delayed diagnosis, which is an independent risk factor for poor prognosis [21,22]. Secondly, the metaphyseal cartilage is not yet mature in infants and infections can spread to the bone marrow via the metaphyseal vessels and develop osteomyelitis, which is another risk factor for poor prognosis [22]. Early diagnosis and treatment (irrigation of the joint cavity and antibiotics tailored to the pathogen) are crucial to achieve long-term satisfaction. However, even MRI does not allow early diagnosis, which is the most recommended in septic arthritis. In addition, PCR is recommended to improve the detection rate of pathogenic microorganisms [23]. The satisfactorily low detection limit of ddPCR is of greater importance for improving the detection rate of pathogens, which is particularly suitable for septic arthritis in infants, and is promising to achieve early pathogens diagnosis.

ddPCR has an overall higher detection rate in patients who have previously received empirical antimicrobial therapy. Culture performance is affected by prior antibiotics. Although ddPCR was also impaired, the effect was significantly less than that of culture. In a study of bloodstream infections, ddPCR still had a sensitivity of 55.56 % and a specificity of 78.75 % in patients who had previously received antimicrobial therapy [24]. In addition, the sensitivity is higher than other nucleic acid detection methods such as qRT-PCR [5]. mNGS achieves wide coverage and has been widely used in patients with FUO. However, there is a possibility that pathogens may not be detected because mNGS is unable to detect trace amounts of nucleic acid in the blood. A study on bloodstream infections has shown that the detection rate of ddPCR within the detection range of ddPCR targets is significantly higher than that of mNGS [6].

Another advantage of ddPCR is the detection of polymicrobial infections. Many types of infections are polymicrobial, and more than one species is involved at the site of infection, including bone and joint infections. Approximately 7.5 % of osteomyelitis of the spine, 60 % of septic arthritis of the knee, and up to 78.6 % of chronic osteomyelitis of the femoral are polymicrobial [25–27]. However, the detection of multiple microorganisms is difficult in culture because the different species vary in difficulty to culture and some dominant bacteria appear during the culture process. As a result, culture often detects fewer pathogens than are actually present. ddPCR is a detection method that is independent of culture and has been shown in previous studies to be highly advantageous over culture in detecting polymicrobials, which is crucial for appropriate treatment, improving prognosis, and reducing medical costs [26]. For example, in patients with bloodstream infections, ddPCR showed a higher rate of polymicrobial infections than blood cultures (38.3 % versus 8.0 %) [24]. In a study of patients with pleurisy, ddPCR demonstrated a higher proportion of polymicrobial infections than bacterial culture in pleural effusion [28].

It is also interesting to note that ddPCR can detect pathogens in peripheral blood samples of patients with local infections. This is of great significance in patients with FUO, as it is often difficult to find definite foci of infection, or the lesion is located deep in the body, making local sampling difficult. Broken microbial gene fragments derived from pathogens or dying human cells/tissues are believed to enter the blood from local foci of infection [29]. Fortunately, ddPCR analysis of blood samples can detect trace concentrations of microbial gene fragments to identify the pathogens. Studies have shown that ddPCR is sensitive enough to detect *Mycobacterium*



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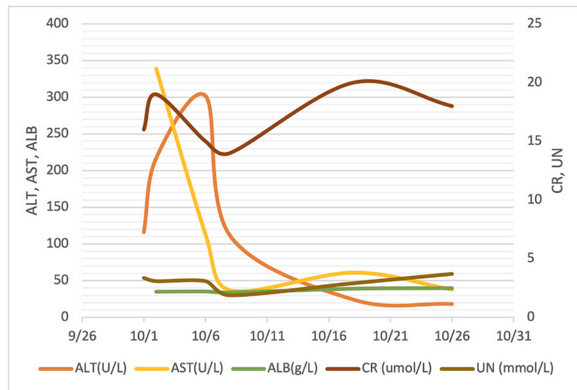

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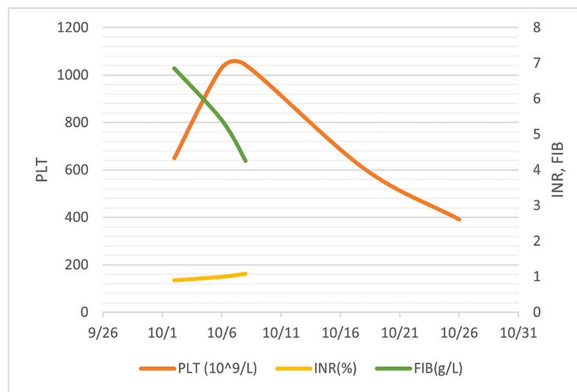

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Fig. 4. Dynamic changes in patient's inflammatory biomarkers and organ function. Fig. 4A shows that inflammatory biomarkers improved after therapy. erythrocyte sedimentation rate, ESR; lactate dehydrogenase, LDH; C-reactive protein, CRP; procalcitonin, PCT. Fig. 4B shows the patient's liver and kidney function. He initially had liver function impairment which improved after infection control. alanine aminotransferase, ALT; aspartate aminotransferase, AST; albumin, ALB; creatinine, CR; urea nitrogen, UN. Fig. 4C shows he had no abnormal coagulation function. blood platelet, PLT; international normalized ratio, INR; fibrinogen, FIB.

Tuberculosis nuclear acid in blood, which can help in the diagnosis of patients with pulmonary tuberculosis and extrapulmonary tuberculosis [30]. In addition, another study showed that ddPCR analysis of blood samples has the potential to monitor the effectiveness of treatment of local infections [31]. Patients with local infections have only traces of cfDNA of the pathogens in their blood, which cannot be detected by other nucleic acid testing methods.

After several days of empirical antimicrobial therapy, this patient still had a fever, and the CRP value was over 100 mg/L. This indicated an unsatisfactory clinical response that was speculated to be the main cause of avascular necrosis of the femoral head. A recent study showed that persistent fever after 48 hours of initial antimicrobial therapy, or a CRP value $\geq$ 100 mg/L 2–4 days after

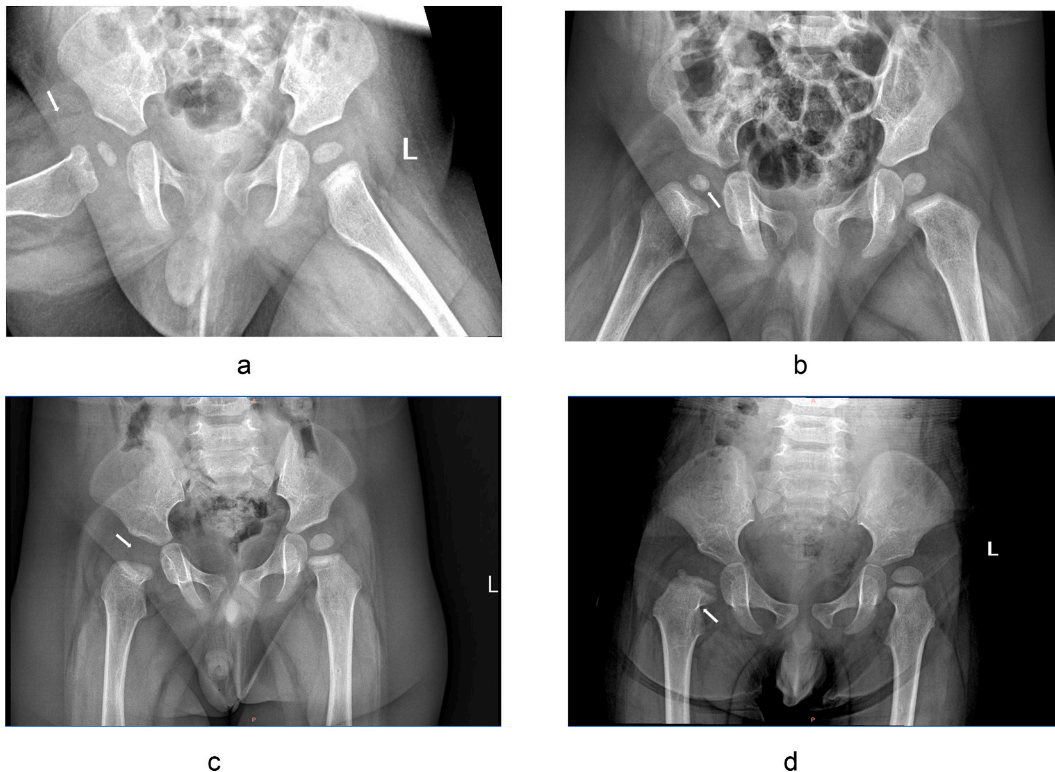


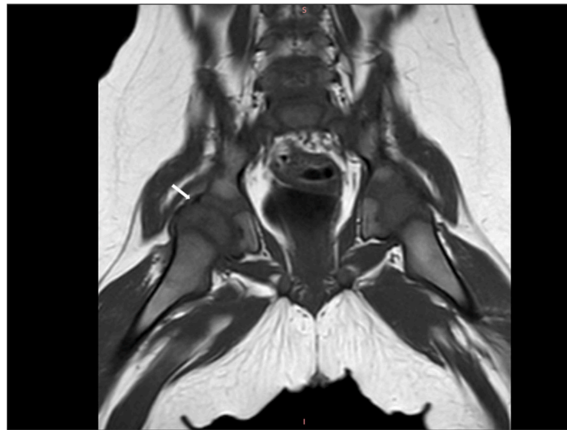
Fig. 5. Dynamic changes in the patient's hip X-ray from one month to one year after surgery. Fig. 5A. Hip X-ray (Nov. 21, 2021) shows swelling and blurring of soft tissues (white arrow marking), destruction of the epiphysis, uneven density, and small shape in the center of secondary ossification of the femoral head, with no signs of bone marrow edema. Fig. 5B. Hip X-ray (Dec. 6, 2021) shows no obvious soft tissue swelling but a further size reduction in the secondary ossification center of the femoral head (white arrow marking). Fig. 5C. Hip X-ray (Feb. 23, 2022) shows no obvious swelling in soft tissues butthe loss of the secondary ossification center (white arrow marking). Fig. 5D. Hip X-ray (Oct. 31, 2022) shows the shortening of the epiphyseal femoral neck, enlargement of the epiphysis, and reduction of the density (white arrow marking).

initiation of therapy indicated poor therapeutic efficacy and predicted the occurrence of complications [32]. Early targeted antimicrobial therapy is the key to preventing secondary complications. When targeted treatments were given based on the results of the ddPCR analysis, his body temperature rapidly normalized, the various inflammatory biomarkers improved dramatically, and the inflammation signs faded on imaging, indicating the value of ddPCR in antimicrobial drug selection. However, it was unfortunate that sequelae remained due to the delay in targeted treatment. During the treatment, the child's parents appreciated the discovery of causative pathogens through the ddPCR analysis and timely antimicrobial drug adjustment, which had a good clinical response. However, the sequelae still beset the child and his parents. This case provides reference value: in febrile patients with an unknown cause, early pathogen screening with the ddPCR assay may help ensure effective therapy's timely usage, which could avoid poor outcomes.

This study has the following limitations: First, it is only a single application in a child with FUO, and the authenticity and reliability of this panel cannot be determined. Therefore, it has only a limited reference value. In the future, more cases need to be included to demonstrate the value of ddPCR. Secondly, the ddPCR panel we developed can only detect some common pathogens, and other tests, such as mNGS, are needed for infection with rare pathogens in children. It cannot replace traditional detection, but it can serve as a complementary tool. Due to the complex etiology of infectious pathogens for FUO, we can also develop other ddPCR panels for rare pathogens to meet clinical needs in the future. Thirdly, it is unfortunate that this patient was not in the early stage of the disease at admission, but that sequelae were still present despite targeted anti-infectious therapy. We advocate the use of the ddPCR analysis as a routine test for children with FUO in community and local primary hospitals, which is crucial for early diagnosis and intervention. In the future, we will include more successful cases to demonstrate the value of ddPCR.

5. Conclusions

As a fast and accurate pathogen detection method, ddPCR can be applied to the diagnosis and guidance of treatment in children with FUO. It has the advantages of high sensitivity, the ability to detect polymicrobial infections simultaneously, and the ability to detect local infections through blood samples. In febrile patients with an unknown cause, early pathogen screening with the ddPCR assay may help to effective therapy, which could be expected to improve prognosis. ddPCR helps to identify pathogens in the diagnosis



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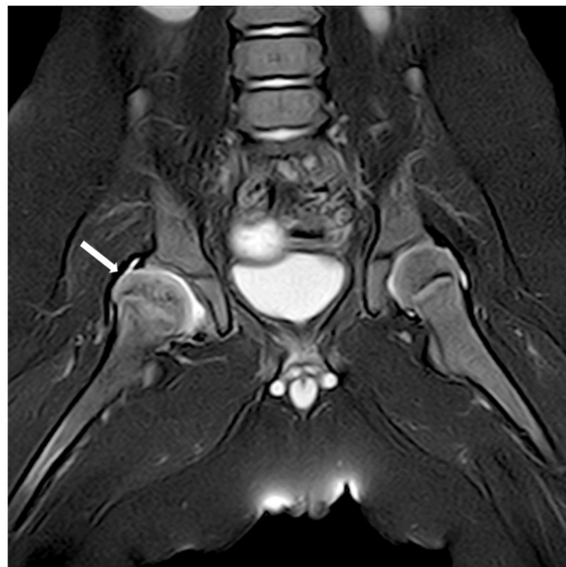

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Fig. 6. The patient's hip magnetic resonance imaging three months after surgery (Feb. 27, 2022) shows a predominantly cartilaginous component in the femoral epiphysis with uneven signal and absence of an ossification center (white arrow marking). Fig. 6A shows the T1 weighted sequence, and Fig. 6B shows the T2 weighted sequence.

of children with FUO and could be a promising complementary tool.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

CRediT authorship contribution statement

Ying Yang: Writing – original draft, Methodology, Conceptualization. **Chunzhen Hua:** Writing – review & editing. **Yan Liu:** Writing – review & editing, Validation. **Cheng Yang:** Data curation. **Yumei Mi:** Visualization, Investigation. **Wei Qiu:** Validation, Software.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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