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Short Communication

Next Generation Sequencing of Free Microbial DNA for Rapid Identification of Pathogens in Critically Ill Children with Systemic Inflammatory Response Syndrome (SIRS)

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Abstract

Background: Infections, major surgeries, and hyperinflammatory syndromes are known to trigger Systemic Inflammatory Response Syndrome (SIRS). Discrimination between infectious and noninfectious inflammation often poses a challenge in chronically ill patients with multiple comorbidities. These patients are routinely treated with a variety of anti-infective medications before a pathogen is identified. With the goal of improving pathogen detection rates and interventions, we evaluated Next Generation Sequencing (NGS) as a highly sensitive and fast means of detecting free microbial DNA in a small amount of serum samples from children with ongoing SIRS. **Methods**: We describe seven complex pediatric patients of SIRS or prolonged fever (>38.5 °C) >72 hours in which serum samples analyzed by NGS had a major impact on therapy. One patient was analyzed twice. **Results**: In eight NGS there were six positive results (two bacterial, three viral, one fungal) which were subsequently confirmed by microbiological culture or polymerase chain reaction (PCR) in five of the six NGS. In five of the eight performed NGS, results led to a change of therapy: antibiotic therapy was discontinued in two, escalated in one, an initiated in another; in one an antiviral was administered. **Conclusions**: NGS may become a valuable addition to infectious disease diagnostics in cases of pediatric SIRS. However, NGS has not yet been validated as a diagnostic method in pediatric as a diagnostic method in pediatric patients and results should therefore be interpreted with caution. Multi-center NGS evaluation studies are currently being planned.

Keywords: SIRS; children; next generation sequencing

1. Background

Systemic inflammatory response syndrome (SIRS) in children is defined by the presence of two out of four clinical criteria, including elevated or depressed leukocyte count, irregular body temperature, tachy- or bradycardia and elevated respiratory rate. Abnormal leucocyte count or temperature are obligatory features1. Infections, major surgeries, and hyperinflammatory syndromes are the most frequent triggers of SIRS [1,2]. In patients with complex underlying diseases, specific infectious symptoms are often absent due to immunosuppression or overlap with features of the underlying disease. In these cases, SIRS is often treated with a variety of anti-infective medications, which may confound diagnosis and yield false negative results. Results also remain negative in cases of noninfectious inflammation, lack of method sensitivity, preanalytical errors, or non-cultivable/fastidious germs. Discrimination of different causes of SIRS is challenging especially in immunocompromised patients since opportunistic infections have to be taken into consideration and usually need to be addressed by targeted diagnostic procedures.

The clinical course of SIRS depends on timely initiation of adequate diagnostics and therapy. The infectious work-up routine includes culture-based methods, nucleic-acid-based technologies (NAT), polymerase chain reaction (PCR), and antigen assays, which all have limitations. Culture-based methods are time-consuming and prone to pre-analytic errors (e.g., contamination, delayed processing, or insufficient specimen) [3]. Targeted NAT and antigen assays are usually faster but are limited to the targeted pathogens and give no or incomplete information on antibiotic resistance. PCR is limited as it can only analyze prede-

Table 1. Summary of NGS results in critically ill children with SIRS of unknown etiology.

Summary of NGS results in critically ill children	
Total number of patients with serum samples analyzed by NGS	7
Total number of NGS analyses performed	8
Male	4 (57%)
Female	3 (43%)
Age (years) [median (range)]	10 (8–16)
Underlying disease	
Hematopoietic malignancy	4 (57%)
Cystic fibrosis	1 (14%)
PIMS-TS	1 (14%)
Granulomatosis with polyangiitis	1 (14%)
Time to NGS results (days after blood collection) [median (range)]	3 (2–4)
Positive NGS findings	5 (63%)
Negative NGS findings	3 (37%)
Therapy adjusted based on NGS	5 (63%)
NGS result matched the established/ standard diagnostic test result	7 (88%)
NGS result did not match the established/standard diagnostic test result	1 (12%)

fined microbes.

Despite proper conventional infectious workup including application of the above-mentioned tests, the causes of SIRS in a complex pediatric case may not be identified. Recently, next generation sequencing (NGS) of free microbial desoxyribonucleic acid (DNA) in blood was evaluated in adults with sepsis [4]. This method increased the number of identified pathogens compared to standard diagnostic care. In contrast to conventional PCR-based results, NGS is not limited to the identification of a predefined list of suspected species [5].

In this study we sought to investigate the diagnostic value of NGS in children with SIRS.

2. Materials and Methods

In this single-center retrospective study (from November 2020 to August 2021) we evaluated NGS analysis of serum samples from seven pediatric patients with prolonged fever and SIRS. One patient was analyzed twice, in two different hospitalizations due to severe SIRS. SIRS was diagnosed based on the criteria defined at the International paediatric sepsis consensus conference [1]. Two out of four criteria applied and one was abnormal temperature or leucocyte count (core temperature $>$38.5 °C or $<$36.0 °C, tachycardia with heart rate $>$2 standard deviations (SD) above normal for age or otherwise unexplained elevation; mean respiratory rate $>$2 SD above normal for age or mechanical ventilation for an acute pulmonary process; leukocyte count elevated or depressed for age or $>$10% immature neutrophils [1]). The following parameters were collected: patient demographics, clinical findings, and laboratory test results including pathogen diagnostics. Patient demographics are presented as median and range, as well as direct descriptive values, as shown in Tables 1,2.

This retrospective analysis was approved by the local Ethics Commission of the University of Duisburg-Essen (21-10180-BO).

Next Generation Sequencing (NGS)

We used NGS, an unbiased sequence analyses of circulating cell-free deoxyribonucleic acid (cfDNA), as a diagnostic tool for SIRS in a children's hospital. NGS-based diagnostics were carried out as follows: After an aseptic removal 2.7–10 mL patient's blood was drawn into two Streck Cell-Free DNA BCT tubes (Streck, La Vista, NE, USA) containing a cell stabilizer by the treating physicians or nurses. Samples were shipped to the Noscendo GmbH (Duisburg, NRW, Germany) on a cool pack. At Noscendo GmbH, further sample processing took place, which included plasma preparation using a two-step protocol (1st centrifugation at 1600 $\times$ g 10 min at 4 °C, transfer of the supernatant and 2nd centrifugation at 16,000 $\times$ g 10 min at 4 °C) and nucleic acid isolation from plasma using the QIAsymphony DSP Circulating DNA Kit (Qiagen, Hilden, Germany). Quantification and quality controls were performed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific,Waltham, MA, USA) and HS NGS Fragment Analysis Kit using a Fragment Analyzer (Agilent, Santa Clara, CA, USA).

Library preparation was carried out from one ng cfDNA and sequencing was performed on an Illumina NextSeq550 (Ilumina, San Diego, CA, USA) instrument with at least 25 million reads sequencing depth per sample. Bioinformatic analysis was performed with Nosecendo's DISQVER® platform. Briefly, human-DNA was computationally excluded and the remaining non-human DNA was systematically analyzed using a proprietary microbial genome reference database consisting of different sources (e.g., RefSeq) covering more than 16,000 microbes and more than 1500 pathogens. Overall, DISQVER® can identify bacteria, DNA viruses, fungi and parasites, simultaneously. The treating physicians were informed about the analytical results through the NOS-Portal, where they also could access the reports.

Table 2. NGS impact on patient treatment.

Patient Number	Treatment before NGS	NGS Test Result	Standard Diagnostic Test Matching NGS Result	Treatment Adjustment Based on NGS	Outcome at Day 28 post NGS
1	Linezolid, meropenem, cidofovir, adenovirus (ADV)-specific T-cells (second dose), antifungal prophylaxis	Positive for ADV, Human polyomavirus 1 (BK)- Virus 1 day after arriving in laboratory	Positive for ADV by PCR in blood and stool; BK Virus in blood 3 days after arriving in laboratory	None	No signs of BK Virus, continued treatment for ADV Patient after bone marrow transplantation (BMT), transferred to a rehabilitation center
1	Cotrimoxazole (*Pneumocystis jiroveci pneumonia* (PJP)- Prophylaxis), voriconazole, cidofovir, meropenem, and other antibiotics prior to admission in our Pediatric Intensive Care Unit (PICU)	Positive for BK Virus, ADV, and *Aspergillus fumigatus* 1 day after arriving in laboratory	Positive for BK Virus by PCR in blood and urine; ADV by PCR in stool, urine and blood; *Aspergillus fumigatus* by antigen in bronchoalveolar lavage and blood, culture of bronchoalveolar lavage, and PCR in blood and tracheal secretions 2 days after arriving in laboratory	None	Patient died 15 days after NGS due to disseminated ADV infection and invasive aspergillosis
2	Cefuroxime and metronidazole followed by meropenem and vancomycin	Negative 1 day after arriving in laboratory	Negative 1 to 7 days after arriving in laboratory	Discontinued vancomycin and meropenem after 2 more doses	No signs of infection, no signs of viruses, fungi or bacteria Patient transferred in good clinical health to a rehabilitation center
3	Piperazillin, tazobactam, and vancomycin	Negative 1 day after arriving in laboratory	Negative 1 to 3 days after arriving in laboratory	None Planned bone marrow transplantation; patient in aplasia	Another inpatient stays due to febrile neutropenia without known focus
4	Meropenem	Negative 1 day after arriving in laboratory	Negative 1 to 4 days after arriving in laboratory	Discontinued antibiotic therapy	No signs of infection Remained in hospital for adjustment of antihypertensive medication
5	Cotrimoxazole (PJP-Prophylaxis), meropenem, vancomycin, clarithromycin, liposomale amphotericin B	Positive for Humanes-Herpes-Virus-3 (HHV-3) 1 day after arriving in laboratory	Positive for HHV-3 by PCR in blood 2 days after arriving in laboratory	Started acyclovir	no signs of infections, still in the hospital for preparation for the planned bone marrow transplantation
6	Piperazillin/tazoba ctam then escalated to meropenem, vancomycin, and voriconazol	Positive for *Pseudomonas aeruginosa* and *pseudomonas* protegens 1 day after arriving in laboratory	Negative More than 7 days after arriving in laboratory	Added tobramycin	No sign of *Pseudomonas* or other infections Patient could continue with intensive chemotherapy
7	Doxycycline and ciprofloxacin (d 21) than change to meropenem	Positive for *Mycobacterium chimaera* 1 day after arriving in laboratory	Positive for *Mycobacterium chimaera* by culture of blood 2 to 14 days after arriving in laboratory	Started rifampicin, ethambutol, and azithromycin, Removed catheter	No signs of infections or *mycobacterium chimaera* Patient treated on an outpatient basis

3. Results

In our university children's hospital, we performed eight NGS analyses in seven children with SIRS to identify potential pathogens. NGS was performed in four male and three female patients ages 8 to 16 years (median age 10 years). Four patients had underlying hematopoietic malignancies (one acute lymphoblastic leukemia (ALL), one acute myeloid leukemia (AML), two lymphoma); one patient had cystic fibrosis, one respiratory failure and fever in the context of granulomatosis with polyangiitis, and one suffered from Pediatric Inflammatory Multisystem Syndrome temporally associated with SARS-CoV-2 infection (PIMS-TS).

Median duration of fever was 7 days (0–18 days) before NGS was performed. In one patient fever persisted even longer over several weeks. We performed NGS in one patient without a fever due to rapid clinical deterioration despite receiving both broad-spectrum antibiotics and antifungal therapy. NGS results were available within 48 hours after taking blood in five NGS analyses (patient 1 (once), 2–4,6), within three Days for one (patient 5) and four days in another two analyses (patient 1 (once), 7). NGS yielded positive results in five out of eight analyses (63%). In six out of eight analyses (patient 1–5,7) results of NGS were verified by standard/established diagnostic care but were received faster (median 1.8 days (range: –2 – >7 days)) in six out of these eight analyses (patient 1–4,6,7). NGS results led to a change of therapy in five out of eight analyses (patient 2,4–7). In two patients (patient 2,4) with negative NGS results, antibiotic treatment was discontinued without recurrence of SIRS and in three patients' antimicrobial therapy was adjusted due to NGS. In one patient (patient 5) HHV-3 could be detected and antiviral therapy with acyclovir was initiated. In another patient (case 6) two different strains of *Pseudomonas spp.* could be detected leading to additional treatment with tobramycin, after a variety of ineffective treatments. The patients' clinical condition slowly improved, fever discontinued and the patient completely recovered. All NGS analyses are summarized in Tables 1,2.

4. Discussion

In this case series we present the results of NGS diagnostics and its impact on treatment of seven children with prolonged fever of unknown origin or SIRS. In five of eight NGS analyses, NGS yielded positive results (two bacterial, three viral, and one additional fungal) and led to treatment modification. In four positive NGS analyses, NGS results were later confirmed by standard procedures except in one case in which the patient rapidly improved after treatment modification according to the NGS result. This lack of confirmation may be culture-based methods is not uncommon for patients with prolonged aplasia and might be a benefit of the NGS. However, at this point in time, with such a small number of patients, we can neither document nor prove this. Future larger studies will be necessary. Two patients received specific testing for the pathogen identified by NGS (*HHV-3, Mycobacterium chimaera*). In three NGS analyses, no pathogen was identified. One of these patients suffered from PIMS-TS, which is caused by a sterile auto-inflammation. Retrospectively two cases could be explained by rising cells, as it occurs after chemotherapy-induced severe bone marrow-depression, as known in transplantation. In two NGS analyses the treatment was discontinued, sparing the patients possible side effects. Twenty-eight days after performing NGS only one patient suffered an ongoing infection and had to be treated at PICU. Four patients were without therapeutic anti-infective treatment. In seven patients, NGS provided a faster result than standard diagnostic work-up. Should future large prospective studies suggest further benefit for patients, a seven-day availability of NGS testing within a 24-hour turn-around would be desirable.

The present case series shows that NGS expands diagnostic possibilities in children with SIRS and comorbidities. These patients are at higher risk for infections by opportunistic pathogens and thus may particularly benefit from early, reliable identification of the causative pathogen. NGS can identify a wide variety of pathogens (bacteria, fungi and viruses) within a single sample, including pathogens that are difficult to cultivate or are slow-growing such as atypical mycobacteria, which are rarely examined [6]. However, 7 patients are a very small number and larger multicenter cohorts are needed to make valid statements.

By providing an individually tailored therapy based on the detected pathogens, adverse drug events or toxicities can potentially be reduced. Yet, these results need to be interpreted with care, as it remains unclear if a negative result in NGS is reliable, especially in patients with a localized infection like pneumonia or abscess. As NGS detects DNA, an important limitations are infections due to RNA-based Pathogenes. Only one if fit clinically, the diagnostics must be expanded. As a consequence, controlled prospective studies are urgently needed to investigate such uncertainties, especially the safety of discontinuation of anti-infective therapy based on a false negative NGS result.

There are several limitations to the NGS method. First, NGS diagnostics is currently reserved to a few hospitals for evaluation purposes. Depending on availability, it can take several days to obtain results. Moreover, there is no option for phenotypical resistance testing. High costs have to be taken into consideration as well and be balanced against benefits in prospective studies. Currently, NGS might be regarded a promising addition to conventional diagnostics but may not replace them [7]. Our NGS analyses illustrate its potential merit, especially in pediatric patients with a diagnosed SIRS or severe critically ill pediatric patients. A multicenter study on the value of NGS in pediatric sepsis is about to be launched and should provide more reliable data [8].

Author Contributions

SCG collected all samples, as well as the clinical data and was with MS and CDS responsible for writing and editing this manuscript. The primary idea for collecting the data used within the manuscript was contributed by CDS as well as TB, who also contributed to the final changes. SCG and BD was responsible for further literature research contributing to editing the manuscript. NB, ET, SS and FS were responsible for providing clinical data as part of the patient care and contributed to editing the manuscript. PMR and SV were responsible for supervision of the laboratory process at the University hospital Essen and sequencing data. They also contributed to the manuscript by critically reviewing and editing. SG was responsible for supervision of the laboratory process at the NG and sequencing data. She also contributed to the manuscript by writing, critically reviewing and editing. PH collected sample data and contributed to editing the manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of the Medical Faculty of the University Duisburg-Essen (21-10180-BO) and conducted in accordance to the latest version of the Declaration of Helsinki. As the study presents a retrospective analysis with anonymous data the ethics committee waived the need for informed consent.

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Conflict of Interest

The authors declare no conflict of interest. Dr. Grumaz is a co-founder, employee, and shareholder of Noscendo GmbH. Petra Horvatek is an employee of Noscendo GmbH. No specific research funding was used for this research.

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