Title: From days to hours: fast diagnostics of bloodstream infections enabled by metagenomic DNA sequencing

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Background:
Sepsis is a common condition often associated with significant mortality due to the development of septic shock and contributes to a substantial health burden both in Denmark and worldwide. The World Health Organization (WHO) estimates that there are 49 million cases of sepsis and 11 million sepsis-related deaths globally each year, with the incidence increasing [1]. Sepsis is a serious illness, often caused by bacteria entering the bloodstream (bacteremia), but can also be due to viruses or fungi in the bloodstream. Symptoms often include high fever and chills. Bacteremia is confirmed by the growth of bacteria from a blood culture obtained from a patient with clinical signs of infection. The most commonly occurring bacteria are Escherichia coli, followed by Staphylococcus aureus [2]. Especially adults over 65 years and infants under one year have a higher risk of death within the first 30 days after bacteremia [3].

The Department of Clinical Microbiology at Aalborg University Hospital performs microbial diagnostics for the entire North Jutland Region and conducts approximately 28,000 blood cultures annually, but only about 10% are culture-positive. Blood culture, as the name suggests, is a culture-based method that often takes several hours or up to 2-3 days to yield a positive result. All blood cultures are reported by a microbiologist to the treating physician with advice on the significance of the culture findings in relation to the patient's clinical syndrome and antibiotic treatment. However, the treating hospital physician often needs to start empirical antibiotic treatment without waiting for the blood culture results, as it is not feasible to wait for culture results in critically ill patients with fever. This poses a risk of starting either broad empirical antibiotic treatment or inappropriate antibiotic treatment. In a study from the USA and Canada, this resulted in >20% of all patients receiving inappropriate antibiotics, leading to significantly higher mortality [4].

Additionally, it can be problematic and undesirable to treat with 'unnecessary' broad-spectrum antibiotics in an era of increasing resistance development. The WHO estimates that multi-resistant bacteria alone will cause 20% of all deaths by 2050 if we do not change our antibiotic usage practices [5]. Furthermore, there are antibiotic-related side effects, such as Clostridioides difficile infection and a reduction in the diversity of the normal gut microbiota. The sensitivity of a culture-based method can also be significantly reduced if the patient has received antibiotic treatment before the blood culture is taken.

Metagenomic DNA Sequencing as a Diagnostic Alternative
To address the challenges described above, this project will use state-of-the-art DNA sequencing technology and molecular methods developed for the study of ancient DNA to identify bacteria, viruses, or fungi in the blood of patients admitted with sepsis and for whom a blood culture has been ordered by the treating physician. This technology has the potential to revolutionize clinical microbiological diagnostics by reducing the turnaround time from sampling to result reporting to the treating physician to under 6 hours. Additionally, DNA sequencing has higher sensitivity, and DNA from dead bacteria can also be detected. The test result can thus be reported on the same day the sample is received, within a time frame that can significantly impact early diagnosis and allow the treating physician to prescribe fewer inappropriate broad-spectrum antibiotics such as cephalosporins, fluoroquinolones, and carbapenems [6].
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Project description

Study Design:
The study is initiated by the principal investigator in collaboration with the Emergency Department at Aalborg University Hospital, led by Dr. Anne Lund Krarup, and the Center for Microbial Communities at Aalborg University, led by Professor Mads Albertsen.

Patients admitted to the Emergency Department at Aalborg University Hospital on suspicion of sepsis and for whom a blood culture set is obtained will be included in the study. Before the blood culture set is taken, the patient will receive written participant information and an oral explanation of the study. Only if the patient provides written consent will they be included, and an additional blood sample (see below) will be taken from which microbial DNA will be purified and sequenced in batch.

The results of DNA sequencing will then be compared with the negative and positive culture results from the blood culture, and information will be obtained from the patients' medical records to evaluate the diagnostic value of this additional analysis. It is up to the principal investigator and the project team to assess the significance of any microbial DNA mapped that identifies a potential pathogen.

This information is obtained retrospectively after the patient's admission, and they will not be directly informed about it. If there is a positive blood culture, the microbial DNA may naturally show the same microorganism. If there is a negative blood culture but positive DNA sequencing with the identification of a potential pathogenic microorganism, the project team will evaluate whether this could be the cause of the patient's illness.

Power Calculation

The power calculation is based on the following assumptions for the statistical analysis that will be subsequently used:

1) The results are dichotomous (positive or negative). This applies to both blood culture and DNA sequencing, where results can be considered either positive or negative. In case of multiple positive pathogens from one sample, it will still be considered positive.

2) The results are dependent on each other because the same patient undergoes two analyses at the same time. Based on the above assumptions, McNemar's test for statistical significance is used. The following assumptions are used in calculating the number of required inclusions. Generally, about 10% of blood cultures are positive (p0), and the best estimate is that DNA sequencing can detect about 20% of causative pathogens (p1). With a power of 90% and alpha = 0.05 where z1-α2=1.96 and z1-β=1.282, this gives the following sample size:

\[
 n = \left( \frac{\left( z_{1-\alpha/2} \sqrt{p_1 + p_0} + z_{1-\beta} \sqrt{p_1 + p_0 - (p_1 - p_0)^2} \right)}{p_1 - p_0} \right)^2
\]

\[
 n = \left( \frac{1.96 \times \sqrt{0.20 + 0.1} + 1.282 \times \sqrt{0.20 + 0.1 - (0.20 - 0.1)^2}}{0.20 - 0.1} \right)^2 = 312
\]

Since the proportions for detection on which the calculations are based are best estimates regarding DNA sequencing, permission is sought to include 350 patients.
Hypothese

By using metagenomic DNA sequencing, the study can identify potential pathogenic microorganisms in the patient's blood more quickly and with higher sensitivity than traditional blood culture. DNA sequencing results can be provided quickly and may reduce turnaround time, facilitating rapid diagnostics compared to the current culture-based method, and thereby potentially enabling faster and more targeted (narrow-spectrum) antibiotic treatment for septic patients.

Objectives

To investigate acute septic patients for microbial DNA in the blood when the treating physician has ordered a blood culture through the Emergency Department at Aalborg University Hospital. To determine the turnaround time and compare the results of DNA sequencing with the blood culture results.

Inclusion Criteria

- Adult patients aged 18 years or older.
- Patients admitted to the Emergency Department at Aalborg University Hospital on suspicion of sepsis.
- The treating physician in the Emergency Department must have ordered a blood culture.
- Despite infection symptoms, the patient must be awake, coherent, and relevant.
- The patient must reside in the North Jutland Region.

Exclusion Criteria

- Age under 18 years.
- Incapacitated.
- Dementia.
- Severely confused.

Planning:

Inclusion of patients will occur from May 1, 2022, to December 1, 2022.

A blood sample from each patient will be frozen at -80 degrees Celsius and then analyzed with DNA sequencing in batch after the patient inclusion period. The analysis of all samples will be completed within one year from sampling. The results from DNA sequencing, blood culture, and the patient’s medical history will be retrospectively evaluated, and the results of sequencing and blood culture will be compared.

Patients admitted to the Emergency Department at Aalborg University Hospital on clinical suspicion of sepsis/blood poisoning will be included in the study as trial subjects. Only competent patients will receive written participant information followed by oral information from the treating physician in the Emergency Department. Their response to the participant information will be awaited, and upon written consent, an additional blood sample will be taken in connection with the blood culture set. In case of any doubt about inclusion, the treating physician can contact one of the principal investigator Dr. Hans Linde Nielsen, Morten Eneberg Nielsen, or Chief Physician Anne Lund Krarup. Severely septic patients may be so generally affected and confused that they will not be able to consider participation, and thus they will not be asked about participation. The assessment of whether the patient is suitable for inclusion is made by the treating physician in the Emergency Department, and
the assessment is based solely on a clinical judgment of whether it is deemed reasonable that the
patient can receive the oral information. However, this means that there is a certain degree of selection
bias in the patient cohort, which may reduce the study's validity.

If the treating/receiving physician assesses that the patient, despite infection symptoms, is awake and
clear and can be included in the study, the treating/receiving physician will provide the patient with
oral information about the project after handing out the written participant information in the form of:

- The National Committee on Health Research Ethics’ document: "Forsøgspersonens
  rettigheder i et sundhedsvidenskabeligt forskningsprojekt"
- Written participant information.
- Informed consent for participation (S3) – for the principal investigator.
- Informed consent for participation (S3) – copy for personal use.

If the patient provides written informed consent to participate, in addition to the prescribed blood
culture, an extra EDTA blood tube with 9 ml of blood will be taken.
The EDTA blood sample will be taken in the same venipuncture as the blood culture but after the
blood culture is taken. It is also standard procedure to take various blood samples for the Department
of Clinical Biochemistry to measure infection levels, fluid levels, kidney function, etc., in the same
session. It is important that the extra EDTA blood sample is taken at admission together with the
blood culture, as the basis for comparison of results from each analysis (DNA sequencing and culture)
would otherwise disappear.
The EDTA blood sample will follow the blood culture and be transported to the Department of
Clinical Microbiology, where it will be registered with the same local sample number as the blood
culture. The blood culture will be handled as usual in the microbiology laboratory, but the EDTA
blood sample will be placed in a freezer at -80 degrees, see below about the Research Biobank. The
principle is that the samples will not be examined until all samples have been collected.

If the patient wishes for consideration time regarding final participation, they cannot be included in
the study, as the blood culture set should not be delayed.

The patient can always get answers to further questions regarding the project by contacting the
principal investigator or one of the investigators by phone or email.

**Side Effects**
There are no significant side effects associated with participating in the study. The treating physician
has ordered a blood culture, and an additional EDTA blood sample (9 ml) will be taken in the same
venipuncture.

**Endpoints**
To investigate the use of metagenomic DNA sequencing as a method for identifying the pathogenic
microorganism causing the patient’s sepsis/blood poisoning compared to traditional blood culture.
Although DNA sequencing is performed in batches, it will also be evaluated whether the analysis can
be performed in real-time with a quick turnaround time, thereby enabling rapid diagnostics compared
to the current culture-based method. In the long term, it may potentially facilitate faster and more
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targeted (narrow-spectrum) antibiotic treatment for septic patients. The goal of this project is also to
develop the DNA sequencing method so that it can be used on other sample categories, such as joint
fluids.

Research Biobank
As stated above, all included patients will have an additional EDTA blood sample taken. At the
Department of Clinical Microbiology, Aalborg University Hospital, plasma will be separated from
red blood cells and platelets using centrifugation, and the plasma will be frozen at -80°C. Only the
principal investigator will have access to the freezer. Before freezing, the plasma sample tube will be
labeled with the same local sample number as the blood culture set, so the principal investigator can
always link the sample back to the patient via the local laboratory information system (wwLab
Autonik). When all samples have been collected at the end of the project period, the samples (in
batch) will be thawed in the laboratory at the Department of Clinical Microbiology, Aalborg
University Hospital, and DNA will be extracted for later sequencing. Samples will be stored until
December 31, 2027. All samples will be analyzed within one year, but samples from patients with
blood culture-confirmed bacteremia have proven so rare that we will store samples longer to repeat
the analysis after any method optimizations. Any surplus material will not be stored.

Sequencing and Data Analysis
To investigate the presence of pathogenic microorganisms in the blood sample, a DNA library will
be prepared for sequencing using SRSLY Technology (Claret Bioscience) Library Preparation and
sequenced on Oxford Nanopore’s MinION platform with R10.4 or R10.4.1 flow cells. Sequencing
will be performed at the hospital with direct upload of data to a GDPR-secured server where data
processing will take place. Human DNA in the dataset will not be analyzed and will be discarded
according to WHO standards for deletion of human genomic data: https://www.who.int/publications/i/item/9789240018440 After discarding all human DNA
sequences, any microbial DNA sequences will be transferred to Aalborg University's servers where
the microbial DNA will be matched against a comprehensive database of microbial DNA to determine
the organism it comes from.

Consent and Information from Patient Records
Participation in the study is voluntary and collected information will be treated confidentially.
Participating patients can withdraw from the study at any time. Informed consent is obtained from all
patients at inclusion. By giving written consent to participate in the study, the patient also consents
to the principal investigator subsequently obtaining information from the patient's electronic medical
record. The key medical record information that will be obtained includes:

- Lookup in wwLab for the result of the blood culture set taken at the same time as the EDTA
  blood sample.
- Lookup in wwLab to register other microbiological test results that may be relevant to the
  individual patient's disease course, investigation, and treatment.
- Lookup in the EPJ (NordEPJ) to register the patient's age, gender, medication, comorbidity,
  final admission diagnosis, result of biochemical analyses, duration of the admission, and the
  antimicrobial treatment (ATC code J: Agents against infectious diseases for systemic use)
  prescribed during the current admission.
Data Collection and Management

Information about the trial subject is protected under the Data Protection Act and the Health Act (§40), and the project will be reported to the record of all research and quality activities where the North Jutland Region is responsible for data and where personal data is used. (ID number to follow). Additionally, data will be processed in accordance with the General Data Protection Regulation. All sensitive personal data will be continuously entered into REDCap (https://redcap.rn.dk/) and stored in this program. All data, including the microbial DNA profile from the patients' blood samples, will also be treated confidentially and it will not be possible for the patient to get detailed answers about their own blood sample but will receive the final result of the study unless the patient does not wish to receive it.

Ethical Considerations:

The entire basis for the project's implementation is the rapid recruitment of trial subjects. Upon admission to the Emergency Department, the patient will naturally be received by the nursing staff and the treating physician (emergency physician). If the physician finds an indication for a blood culture based on the patient's clinical symptoms, the treating physician will provide oral information about the study and hand out the written participant information. After written consent from the patient, the additional EDTA blood sample will be taken, and the patient will agree to the other diagnostic tests. An acute admission with fever and possible infection-related symptoms can be a distressing experience for the patient, but it is not considered too psychologically burdensome for the patient to give consent to a blood sample, as this is taken during the same venipuncture as the other diagnostic tests, including the blood culture. To avoid disrupting the patient's course in the emergency department, it is the treating physician who provides the oral information about the study and hands out the written participant information. The patient must be awake, clear, and relevant despite infection symptoms, and it is considered safe for the treating physician who orders the blood culture to assess whether the patient is a candidate for inclusion. If the treating physician is in doubt, they have the option to contact one of the project investigators.

A positive blood culture always prompts a phone call from a physician at the Department of Clinical Microbiology, and it is routine for the microbiologist to collect relevant information from the electronic medical record, such as allergies, clinical symptoms, probable focus of infection, and the prescribed treatment, to provide the best possible clinical advice, including the choice of antibiotic treatment, to the treating physician for the patient's benefit.

The sensitive personal data collected from laboratory analyses, DNA sequencing, and the electronic medical record are naturally covered by confidentiality and will be entered into REDCap. All human DNA will not be analyzed, as it will be deleted without traceability, while any microbial DNA will be clarified and data will be included in the overall cohort of blood-cultured patients in the project period.

Perspectives:

Previous studies of DNA sequencing of blood in infectious patients have shown promising results as a diagnostic method, but there are still challenges with large amounts of human DNA, and sequencing methods can be sensitive to contamination with DNA from the surrounding environment, which is
irrelevant to the infection. We will use new analysis methods developed to study ancient DNA to
enrich microbial DNA to increase sensitivity at a reduced cost. Additionally, Oxford Nanopore is
used as the sequencing platform, which shortens turnaround time from ~30 hours to ~6 hours
compared to previous studies where Illumina sequencing was used. The project's goal is thus to bring
DNA sequencing closer to use in routine clinical microbiological diagnostics, and if the project
succeeds, it will enable a more informed choice of antibiotics for the treatment of patients at Aalborg
University Hospital.

**Publication of Study Results:**
The results of the study will be published at national and international meetings and in a publication
in an international peer-reviewed journal. Both positive, negative, and inconclusive results will be
published. All publication rights belong to the principal investigator. The authorship order will
depend on each individual's contribution to the publication.

**Finance and Budget:**
All involved persons are salaried by their respective employers (AAUH & AAU) and do not receive
additional financial compensation for participating in the trial. Costs associated with DNA sequencing
are covered by already obtained funding from:
- Torben og Alice Frimodts Fond - 15,000 DKK
- Director Jakob Madsen and Wife Olga Madsen's Foundation - 80,000 DKK
- Beckett Foundation - 100,000 DKK
- Harboefonden - 100,000 DKK

Trial subjects will not receive financial compensation for their participation, and no payment is made
for assistance to physicians in the emergency department. Any harm or injury caused to the patient
as a result of the project will be met by a compensation scheme financed by the hospital.
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References:


