

FUTURE DIAGNOSIS OF SEPSIS: EXPLORING THE POTENTIAL OF NANOPORE SEQUENCING AS A DIAGNOSIS TOOL FOR SEPSIS

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Abstract

Sepsis is one of the main causes of death all around the world and could be caused by different types of infections, like viral, bacterial or fungal. Time is of essence when a patient goes septic, usually leaving a few hours only to deliver appropriate treatment. As antimicrobial resistance is becoming more common in recent years, the right treatment being delivered on time is important. Current culture-based diagnosis methods could take 24-72 hours for results and often that might be too late. This is why usually a preliminary treatment is delivered until results are back and this could be not effective if antimicrobial resistance is present in the ongoing infection. That is one of the reasons that researchers have chosen to explore and study other methods like metagenomic Next-Generation Sequencing where DNA is extracted directly from bodily fluid or tissue sample and sequenced and then analyzed. In this study urine from a healthy volunteer was collected and then spiked with different types of spike-ins. DNA was then extracted using two kits, and the DNA extracted was sequenced using the nanopore sequencing technique. The data was then analyzed using EPI2ME software. The results showed the potential this technique can have in clinical applications. In higher spike-in concentrations all expected bacteria species were identified, but not all genes expected that could confer antimicrobial resistance. The results of the experiment showed the significance of the DNA extraction step as the shearing of DNA during extraction lead to shorter and lower quality sequencing reads. This approach opens new doors to healthier communities, equality and sustainability. However, further research and exploration are needed to improve this approach and make it more reliable for clinical applications.

List of abbreviations

EBSL	Extended-spectrum beta-lactamase
ICU	Intensive Care Unit
IQR	Interquartile Range
mNGS	Metagenomic New Generation Sequencing
NGS	New Generation Sequencing
ONT	Oxford Nanopore Technologies
PCR	Polymerase Chain Reaction
QDU	Quick-DNA Urine Kit
qPCR	Quantitative Polymerase Chain Reaction
QUP	QIAamp UCP Pathogen Mini
SD	Standard Deviation
SR	Sequencing Run
UTI	Urinary Tract Infection

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1 Introduction

Sepsis is a life-threatening condition caused by a dysregulated immune response to an infection and it can cause damage and injury to own organs and untreated it could lead to death (Charoensappakit et al., 2023; Shemirani et al., 2023). About 49 million cases of sepsis and 11 million sepsis-related deaths which are 20% of all deaths globally were recorded in 2017, of which, around 85% of them happened in low- and middle-income countries and 50% of all sepsis cases occurred in children (Ali et al., 2024). Sepsis burdens the healthcare system economically because of the need for expensive treatments, ICU stays, and in many survivors, there could be life-long side effects and complications that impact patients and the healthcare system (Avershina et al., 2023; Yamamoto et al., 2025). Urinary tract infections (UTI) are one of the leading infections that could lead to sepsis, and *Escherichia coli* (*E. coli*) is one of the main causes of UTIs (Bellankimath et al., 2024; Isberg et al., 2024; Tilevik et al., 2022).

UTIs are one of the most prevalent infections worldwide with 405 million cases in 2019, with around 236,000 deaths linked to these infections, which is a 60% rise in cases and 140% rise in deaths when compared to numbers from 1990, UTIs can develop into a kidney infection and/or urosepsis which accounts for 25% of all sepsis cases (Bellankimath et al., 2024). *E. coli* is present in around 60% of urine cultures that test positive when it comes to UTIs, both community and healthcare-acquired UTIs, other pathogens like *Enterococcus faecalis* and *Staphylococcus aureus* are majorly present in more complicated infections (Isberg et al., 2024). Two main factors that cause UTI to lead to a patient becoming septic are that the infection is polymicrobial which requires more advanced methods for timely diagnosis, more complicated treatments and care, and another is antimicrobial resistance in infectious agents that challenges clinicians when using empirical treatments (Bellankimath et al., 2024).

Antibiotic resistance related deaths in 2019 were around 1.27 million directly, and around 5 million indirectly and if nothing changes following the same trajectory antimicrobial resistance will be the cause of more than 10 million deaths by 2050 (Avershina et al., 2023). Through unnecessary use of antibiotics and excess exposure to antibiotics, a wide range of bacteria have developed and evolved to survive in the presence of variety of antibiotics (Avershina et al., 2023). As antibiotic resistance becomes more common with infections, the importance of detecting bacterial genes that could confer antimicrobial resistance becomes clearer and developing new diagnosis methods helps minimize the risks and slow down antibiotic resistance (Shemirani et al., 2023). Antibiotic susceptibility tests are done after the bacteria culturing is done, meaning the patient will lose at least 24-48 hours before the antibiotic susceptibility test results return (Carpenter et al., 2025).

1.1 Current and Future Diagnosis

The main method used globally to detect bacterial infection, and the type of infection is by collecting blood or urine depending on the type of infection and growing it in bacteria broth media and when growth is detected the specific bacteria will be detected using

various biochemical tests and MALDI-TOF mass spectrometry (Ali et al., 2024; Bellankimath et al., 2024; Yagupski & Nolte, 1990). Some of the routine biochemical tests conducted to help identify bacterial species in a culture are catalase, oxidase and urease tests where bacteria are analyzed by their ability to break down certain compounds (Hafezi & Khamar, 2024). Antibiotic susceptibility testing in Europe is done using the disc diffusion method in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2025).

These current diagnostic methods that are employed in most health centers, hospitals and clinics take 2-4 days for blood cultures and 1-2 days for urine cultures, and for a potentially septic patient with symptoms survival chances are lowered by 7.6% with every hour that passes without antibiotics being delivered (Ali et al., 2024; Carpenter et al., 2025). Some molecular methods like PCR and qPCR can be used as well but using PCR or qPCR as a diagnostic method is not ideal either because it is only able to identify predefined common infectious agents and not those rare, uncommon ones (Kim et al., 2025; Carpenter et al., 2025).

A challenging situation is when an infection occurs where multiple microbial, viral or fungal agents are active in the infection and where this could lead to complications with the use of antibiotics, misdiagnosis of certain agents or some not being diagnosed at all (Bellankimath et al., 2024; Gosiewski et al., 2017; Jiang et al., 2025; Tilevik et al., 2022). Certain pathogens like extended-spectrum beta-lactamase (ESBL)-producing *E. coli* are resistant to typically used antibiotics, which could challenge treatment prior to diagnosis results that could end fatally (Ali et al., 2024; Isberg et al., 2024; Tilevik et al., 2022). The patient also plays a vital role in effectiveness of treatments as elderly patients with complications like diabetes and kidney diseases are more likely to be affected by UTIs that could lead to sepsis, and they could have different immune responses (Charoensappakit et al., 2023; Gosiewski et al., 2017; Isberg et al., 2024; Yamamoto et al., 2025).

The use of metagenomic Next Generation Sequencing (mNGS) as a diagnostic tool for detecting bacteria species in UTIs has started to gain attention and is being explored (Bellankimath et al., 2024). mNGS is a sequencing approach where genetic material of possibly multiple infectious agents, DNA and RNA, are extracted and multiple strands are sequenced at the same time which will enable unbiased sequencing of DNA material (Huang et al., 2023). As the use of mNGS methods is being explored, the need for adequate data analysis becomes larger as well with parallel exploration of bioinformatics to aid with the speed of diagnosis, where the use of machine learning is also discussed (Avershina et al., 2023). One of the most important challenges with diagnostic methods that employ sequencing are their high cost, expertise needed to perform the method and analyze the data, and resistance by clinicians to adopting these new methods that need to be developed further and standardized more (Avershina et al., 2023; Bellankimath et al., 2024; Jiang et al., 2025; Kim et al., 2025). The concept of nanopore sequencing has existed for a few decades and became commercialized by Oxford Nanopore Technologies (ONT) when ONT released a device called MinION. MinION can identify different DNA bases by measuring changes in electrical conductivity as a DNA strand is passing through a nanopore and MinKNOW, the software used to operate the device can receive the data

sent by the MinION device and base-call it in real-time while the sequencing is happening which can offer faster results (Lu et al., 2016).

1.2 Aim

The future threats and the present problems all point towards the necessity of new diagnostic methods being explored, researched and developed. The new diagnostic methods are important to be early, precise and reliable, alongside they must be standardized in a way that they can be employed in practice in clinics with ease and be as simple to exercise as possible, otherwise their practicality is in question, and health practitioners will be hesitant to adopt these new methods.

The aim of this study was to explore and evaluate the possibility of using nanopore sequencing as a future diagnostic tool for sepsis. To achieve this, a multi-step workflow was created to extract DNA from spiked urine, sequence the extracted DNA on the MinION device, analyze sequencing data and evaluate the capabilities of this workflow to identify pathogens and antimicrobial resistance genes. A secondary goal of this project was to compare the performance of two DNA extraction kits used regarding the sequencing. The research question for this study is whether the sequencing data collected in this experiment is reliable enough for this method of pathogen identification to be used clinically.

2 Materials and methods

2.1 Ethical Considerations

The urine used for the project was self-extracted by a consenting volunteer. The identity of the volunteer was not recorded, so the data collected in this project will remain anonymous. All biological and chemical waste was handled according to local guidelines of the University of Skövde (Thilander et al., 2019). All the data and material collected in duration of this study are stored at the University of Skövde.

2.2 Overview of the study

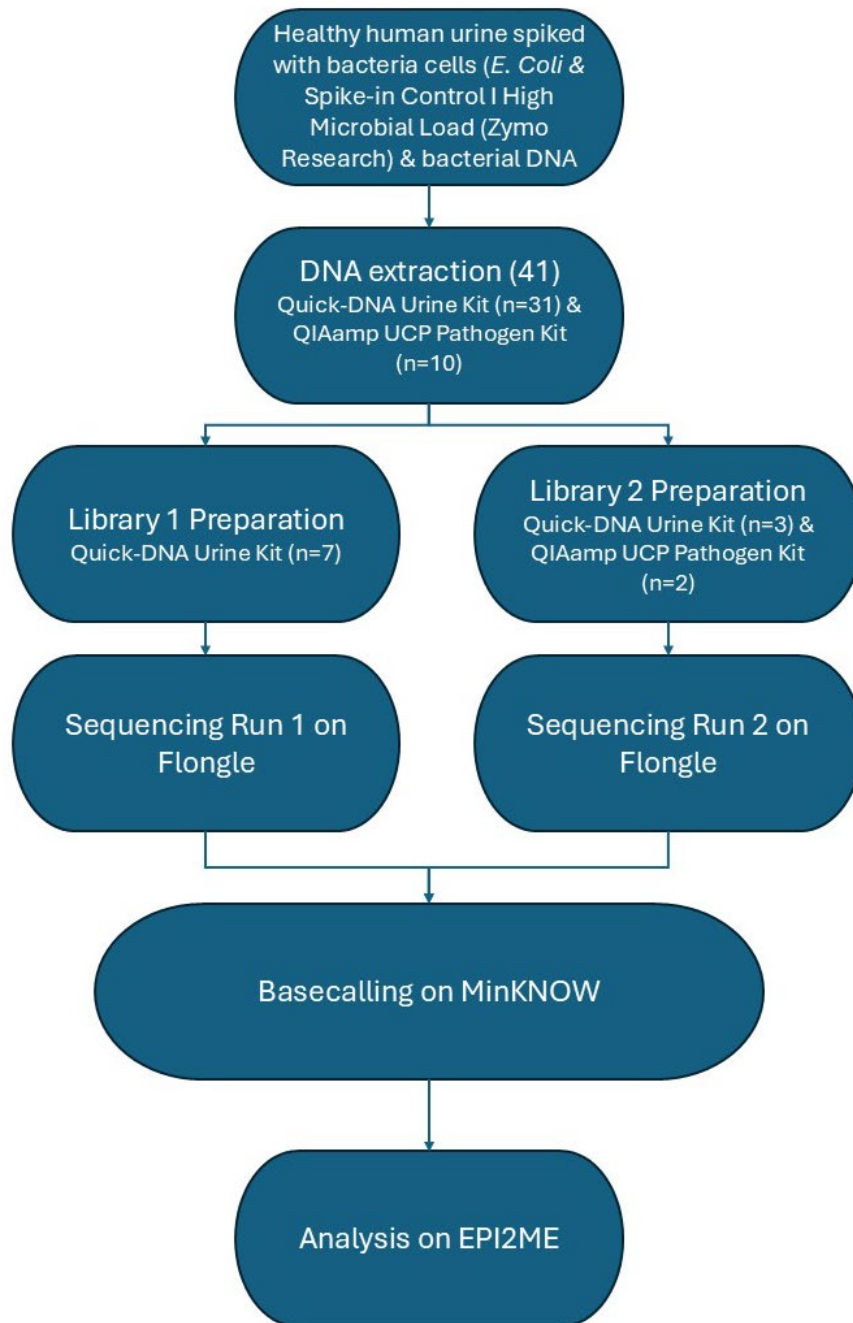


Figure 1. Overview of the procedure: Healthy human urine was collected and spiked with three different spike-ins used in combinations or on their own. DNA was extracted from the spiked urine using two kits (n=41) and underwent quality and quantity control. Then the DNA libraries were prepared for sequencing. The DNA library was sequenced on MinION device using Flongle flow cells. The data was base-called on MinKNOW software and analyzed on EPI2ME software. The analysis included taxonomic classification and the detection of genes conferring antimicrobial resistance.

2.3 Spike-in Material and Preparation

There were three types of spike-ins used in this project to simulate an ongoing infection in urine or the urinary tract. The spike-ins used are Spike-in Control I High Microbial Load (Zymo Research), *E. coli* (Strain: atcc 25922) and DNA (DNAour). Control I spike-in composition included *Imtechella halotolerans* & *Allobacillus halotolerans*. DNAour was extracted locally from bacterial isolates that were collected during a prospective observational study of community-onset severe sepsis and septic shock from patients at Skaraborg Hospital in western Sweden in 2011-2012 (Ljungström et al., 2019). DNAour extracts that were used include DNA from the genus *Haemophilus*, *Corynebacterium*, *Enterococcus* and *Moraxella*. These spike-ins will be referred to as Control I, *E. coli* spike-in and DNAour respectively. The amount of spike-in will be referred to accordingly for convenience: 1X Control I = 2×10^7 CFU, 1X *E. coli* = 2.16×10^7 CFU. In the later experiment 2X to 16X amounts of spike are used. For all steps where water is used, the water was obtained from Milli-Q® SQ 200 Purification System (Sigma-Aldrich).

2.3.1 *E. coli* spike-in preparation in-house

One colony of the *E. coli* (Strain: atcc 25922) was incubated in 5 ml LB-media overnight at 37°C and shaking at 250 RPM in a 15 ml falcon tube. 1 ml of the 5 ml overnight culture was diluted with 24 ml of pre-warmed LB media in a conical flask and was incubated at 37°C while shaking at 170 RPM. The first 100 µl of the culture was taken before incubation (T₀) and thereafter every hour at T₁, T₂...T₆. The 100 µl taken each hour was serially diluted with 0.85% saline water to make 10^{-1} - 10^{-9} dilutions. Then 100 µl of the tubes ranging from 10^{-5} - 10^{-9} were taken and plated on pre-warmed LB-Agar plates in triplicates and then incubated at 37°C overnight. The next day plates containing 30-300 distinct colonies were used to determine the CFU/ml of the culture at each time point and to obtain the concentration of each dilution series' tube accordingly.

2.4 DNA Extraction

Two kits were used to extract DNA from the spiked urine, the QIAamp® UCP Pathogen Mini Kit (Qiagen) and the Quick-DNA™ Urine Kit (ZYMO Research). The DNA extracted were stored in +4° Celsius, like the urine.

2.4.1 Extraction using QIAamp UCP Pathogen Mini Kit (Qiagen)

The QIAamp UCP Pathogen Mini Kit (Qiagen) was used with mechanical lysis to extract DNA from spiked urine samples (Table 1). The elution was done twice, where the initial eluted volume was taken and placed on the column and then centrifuged for a second time.

Table 1. 2 ml urine volume in some extractions was when the pathogen lysis tubes were loaded twice.

Spike	Urine Volume (ml)	Elution Volume (µl)	n
none	1	50	2
4X Control I (Zymo Research)	1 & 2	50	2
8X Control I (Zymo Research)	2	50	2
8X Control I (Zymo Research) + 100 ng DNAour (60 ng <i>Haemophilus</i> & 40 ng <i>Corynebacterium</i>)	2	50	4

2.4.2 Extraction using Quick-DNA™ Urine Kit (ZYMO Research)

The Quick-DNA™ Urine Kit (ZYMO Research) was used to extract DNA from spiked urine samples (Table 2). When taking out the supernatant after total DNA precipitation, 200 µl of pellet was left. To increase DNA yield and protein digestion, the incubation period for proteinase K was increased to 60 minutes. All centrifugation steps in the DNA Purification section were done at 14,000 RCF for 2 minutes, additionally in elution step the duration was 5 minutes. The elution was done twice, where the initial eluted volume was taken and placed on the column and then centrifuged for a second time.

Table 2. Extractions done with the Quick-DNA™ Urine Kit (ZYMO Research). Urine volume for all extractions was 1 ml.

Spike	Elution Volume (µl)	n
*16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	20	3
16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	50	3
8X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	50	3
2X <i>E. coli</i> + 2X Control I	50	3
4X <i>E. coli</i>	50	3
4X Control I	50	3
**4X Control I	50	2
**2X Control I	50	2
8X Control I + 105 ng DNAour (<i>Corynebacterium</i>)	50	3
8X Control I	50	3
none	50	3

*The 3 extractions that were eluted in 20 µl of Ultra-Pure water, the rest of the extraction were eluted using 50 µl. **The incubation period for proteinase K was 30 minutes.

2.5 Quality and Quantity Control of the Extracted DNA

The quality of the DNA extractions was measured using a DS-11 Spectrophotometer (Denovix). The quantities of the DNA extractions were measured using a Qubit Fluorometer (Thermo Fisher) using the Qubit dsDNA 1X High Sensitivity Assay Kit (Thermo Fisher), following the Qubit™ 1X dsDNA HS Assay Kits User Guide (Thermo Fisher, 2020).

2.6 Library Preparation and Sequencing

The extractions (Table 3 & 4) used for generation of DNA libraries (n=2) were chosen based on their spike-in composition, quantity and quality. The DNA libraries were prepared using the Rapid sequencing DNA V14 – barcoding Kit for Flongle SQK-RBK114.96 (Oxford Nanopore Technologies, 2025) and following the handbook (Oxford Nanopore Technologies, 2025). Instead of a thermal cycler two heat blocks were used for enzyme activation and stopping the enzyme.

The sequencing was run using Flongle (FLO-FLG114) flow cells (Oxford Nanopore Technologies), the MinION MK1D (Oxford Nanopore Technologies) device and MinKNOW V25.03.9 (Oxford Nanopore Technologies) software to control hardware, flow cell status and sequencing status, in addition to reading and base-calling the data. The minimum Q-score to pass reads was set to 7.

Table 3. Extractions used for sequencing run #1. Last row was a control. QDU is an abbreviation for Quick-DNA Urine kit (Zymo Research). The raw quantity and quality values of each QDU extraction can be found in appendix II (Table 10).

Biological Fluid	Extraction Number	Extraction Kit	Spike-In
Urine (1 ml)	14	QDU	4X <i>E. coli</i>
Urine (1 ml)	15	QDU	4X <i>E. coli</i>
Urine (1 ml)	11	QDU	2X <i>E. coli</i> & 2X Control I
Urine (1 ml)	5	QDU	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)
Urine (1 ml)	9	QDU	8X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)
Urine (1 ml)	31	QDU	-
Urine (1 ml)	2	QDU	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)
-	-	-	DNAour (<i>Moraxella</i>) control

Table 4. Extractions used for sequencing run #2. QDU is an abbreviation for Quick-DNA Urine kit (Zymo Research) & QUP is an abbreviation for QIAamp UCP Pathogen Mini Kit (Qiagen). Extraction 28 had 18 ng/μl added DNAour (*Enterococcus*) post extraction to be used as control. The raw quantity and quality values of each QDU extraction can be found in appendix II (Table 12). The raw quantity and quality values of each QUP extraction can be found in appendix III (Table 11).

Biological Fluid	Extraction Number	Extraction Kit	Spike-In
Urine (2 ml)	1	QUP	4X Control I (Zymo Research)
Urine (2 ml)	8	QUP	8X Control I (Zymo Research) + 100 ng DNAour (<i>Haemophilus</i> & <i>Corynebacterium</i>)
Urine (1 ml)	28	QDU	4X Control I (Zymo Research) + DNAour (<i>Enterococcus</i>)
Urine (1 ml)	30	QDU	None
Urine (1 ml)	20	QDU	8X Control I (Zymo Research) + 100 ng DNAour (<i>Corynebacterium</i>)

2.7 Statistics Analysis and Tools

To determine if the data was normally distributed python 3.11 and the Scipy and Numpy libraries with a p-value of 0.05 were used, the code used to do this can be found in Appendix I, Figure 2. Mean/Median and SD/IQR were calculated using Excel functions.

The sequencing data was analyzed using the EPI2ME (ONT) Desktop V5.2.3, where the Metagenomics workflow was employed using default settings. This tool uses Kraken 2 (Wood et al., 2019). EPI2ME was used to check for genes that could confer antibiotic resistance against the Resfinder database.

3 Results

3.1 DNA Extraction

For extracting DNA from spiked and non-spiked urine samples, two DNA extraction kits were used.

3.1.1 QIAamp UCP Pathogen Kit (QIAGEN):

The DNA extraction concentrations using the QIAamp UCP Pathogen Kit (Qiagen) ranged around 1 ng/ μ l and less, below the value recommended for DNA library preparation, which was 20 ng/ μ l. The raw concentrations and purity values can be found in Appendix III, table 11.

3.1.2 Quick-DNA Urine Kit (ZYMO Research)

The Quick-DNA Urine Kit showed higher concentrations compared to QIAamp UCP Pathogen Kit when extracting DNA from spiked and non-spiked urine. The concentration values using this kit ranged from around 1 ng/ μ l to 18 ng/ μ l which came close to the 20 ng/ μ l target for DNA library preparation. The raw concentration and purity values can be found in Appendix II, table 10.

3.2 Sequencing & Library preparation

Sequencing runs (SR) 1 & 2 statistics (Table 5) show information about the sequencing; number of reads, and the portion of passed reads based on the Q-score threshold (=7). The quality and quantity control results (Table 5) are relevant to the sequencing runs. Library quality and concentration were slightly higher for SR1 where higher quality and higher quantity of DNA extractions were used. SR1 also showed a slightly higher number of reads. The N50 value was higher for SR2.

Table 5. Sequencing results and statistics along with qualitative library data.

	Duration of the run	Total Reads (Passed Reads)	N₅₀*	Library Concent ration (ng/μl)	Library 260/23 0	Library 260/280
SR1	21:23:00	165.99k (141.61k)	2.2 kb	79	2.31	1.80
SR2	21:34:00	151.46k (126.91k)	6.14 kb	40.2	2.78	1.71

* N₅₀ is a weighted median, and it means that 50% of the reads had a length of 6.14/2.2 kb or higher.

3.3 Metagenomic Analysis

The post-sequencing metagenomic analysis produced the results in table 7 & 8 where the number of reads associated with identified species in each relative extraction can be seen. Average read length for SR2 was higher than SR1, where the quality-scores and proportion of classified reads are relatively similar (Table 6).

Table 6. Classified reads percentage, average q-score and average sequence length.

Parameter	SR1	SR2
Classified reads%	54	47
Q-score	10	10
Average sequence length	604 bases	988 bases

Table 7. Metagenomic analysis results for SR1. Species VS Extraction codes (Kit + Extraction Number). The values in the table represent reads.

Species	QDU 14	QDU 15	QDU 11	QDU 5	QDU 9	QDU 31	QDU 2	DNAour Control
<i>Homo sapiens</i>	6,642	4,349	3,025	2,129	3,702	3,349	2,653	14
<i>Photobacterium leiognathi</i>	17	0	52	24	142	84	23	65
<i>Imtechella halotolerans</i>	0	0	61	0	0	0	0	0
<i>Allobacillus halotolerans</i>	0	0	18	0	0	0	0	0
<i>Escherichia coli</i>	0	0	0	37	0	0	39	0
<i>Enterococcus faecalis</i>	0	0	0	771	1,637	0	739	0
<i>Moraxella (Genus)</i>	0	0	0	0	0	0	0	4,689

Table 8. Metagenomic analysis results for SR2. Species VS Extraction codes (Kit + Extraction Number). The numbers represent reads.

Species	QUP 1	QUP 8	QDU 28	QDU 30	QDU 20
<i>Homo sapiens</i>	100	408	2,049	2,152	11,970
<i>Photobacterium leiognathi</i>	17	24	79	64	108
<i>Imtechella halotolerans</i>	30	333	1,108	0	968
<i>Allobacillus halotolerans</i>	13	185	286	0	299
<i>Enterococcus faecalis</i>	0	0	16,576	0	0
<i>Haemophilus influenzae</i>	0	293	0	0	0
<i>Corynebacterium pseudodiphtheriticum</i>	0	74	0	0	104

3.4 Genes conferring antimicrobial resistance

The EPI2ME software detected genes conferring antimicrobial resistance only in controls, DNAour control and QDU 28 (Table 9). Other extractions showed no detected genes conferring antimicrobial resistance.

Table 9. Antimicrobial resistance found by EPI2ME in Resfinder database. The values represent hits.

Antibiotic Resistance genes	DNAour Control	QDU 28
Lsa(A)	-	24
Tet(M)	-	58
blaBRO-1	5	

The forementioned antimicrobial resistance genes found (Table 9) correspond to the spike-ins as follows: Lsa(A) & Tet(M) to *Enterococcus faecalis* found in QDU 28 and blaBRO-1 to *Moraxella (Genus)* found in DNAour Control.

4 Discussion

In sepsis, where an infection has reached a dangerous state and the body is struggling, time is of essence for the patient, and early diagnosis and treatment are key (Barraud et al., 2019). Therefore, mNGS was chosen to study as a potential future diagnostic tool due to its rapidness and inclusivity for most species compared to conventional methods being used currently in clinics (Carpenter et al., 2025).

When exploring a new method, new challenges arise in each step of the way. To make sure that a successful mNGS is performed, DNA of good quality is needed, and in this case, DNA was extracted from urine. One of the characteristics of urine is its low DNA content, which could make it challenging to obtain suitable amount of DNA while maintaining quality and integrity of the extracted DNA (Vendrell et al., 2022). However, the advantages of using urine are its ease to collect and it being a less invasive way of body fluid sample collection compared to tissue or blood.

To extract DNA with such qualities and quantities, two kits were used and compared to each other. Protocols were modified when needed to suit the project's needs. According to the barcoding kit used for the library preparation, a concentration of 20 ng/ μ l was required in a volume of 10 μ l from each DNA extraction.

4.1 DNA Extraction

The results of the DNA extraction using the QIAamp Pathogen UCP Kit (Qiagen) were far from what was required in terms of quantity (20 ng/ μ l) and quality (260/230: 2.0-2.2 & 260/280: 1.8) which was suspected to be due to ineffective lysis of the cells. This was unexpected since the QIAamp Pathogen UCP Kit (Qiagen) had a mechanical lysis step using tubes with glass beads for better cell lysis. One of the reasons can be an abundance of cell debris from bacteria, which can block the column from binding the extracted DNA successfully. This has been addressed in the troubleshooting part of the

protocols of the Quick-DNA/RNA™ Miniprep Plus Kit (ZYMO Research) and DNeasy Blood and Tissue Kits for DNA Isolation (Qiagen).

Other studies have also shown that maximum effectiveness in extracting tough-to-lyse bacteria DNA in human studies, was achieved when mechanical and chemical lysing tools are used together (Yuan et al., 2012; Lim et al., 2018; Elie et al., 2023). Mechanical lysis is used in this project for the QIAamp Pathogen UCP Kit (Qiagen), but not in the extractions using Quick-DNA Urine Kit (Zymo Research). In another study where urine was spiked with bacteria cells, and five kits were benchmarked, the kits with mechanical lysis steps did not perform best, but one of them even performed worst out of the five (Vendrell, et al., 2022). In the current project the Quick-DNA Urine kit showed higher concentration despite not having any mechanical lysis.

4.2 Sequencing

The sequencing results (Table 8) show an average quality score (Q-score) of around 10 for the sequencing runs in this project. A Q-score of 10 means that there is a 10% chance of error in the base-calling; a higher Q-score of 20 is considered more reliable and standard as it means there is a 1% chance of error (Goswami & Sanan-Mishra, 2022). The relatively low Q-score could be reflective of relatively low-quality DNA libraries (Table 5) which are not either in the ideal range of 260/230 & 260/280 absorbance values of 2.0-2.2 and 1.8 respectively. Also notable to mention that none of the extractions used for sequencing met the concentration threshold of 20 ng/μl that were recommended by the Rapid sequencing DNA V14 – barcoding kit (ONT).

This study used Flongle flow cells for sequencing. Using a Flongle cell instead of a MinION flow cell can have a few advantages and disadvantages. The advantage of using Flongle flow cells (\$166) is their cost effectiveness compared to the more expensive MinION (\$700) flow cell. Another advantage in the context of possible use as a diagnosis tool in the future is that a new flow cell can be used for each test whereas if MinION flow cells were to be used, they would be reused multiple times before they lose their functionality and effectiveness. Reusing a flow cell for diagnosis purposes is disadvantageous because there could be leftover and carryover DNA from previous uses. However, Flongle cells are also considerably smaller than MinION flow cells (approx. 20 times smaller) which means reduced sequencing output and that it is more suitable for smaller libraries. Despite this, the barcoding kit for both flow cells suggests using the same amount of DNA material. Deciding whether to use Flongle flow cells or MinION flow cells depend on the use, the library, the budget and time-sensitivity, either could be suitable for a specific case.

4.3 Metagenomic Analysis

The metagenomic analysis (Tables 7 & 8) successfully detected the presence of *Imtechella halotolerans* and *Allobacillus halotolerans* in every extraction where Spike-in Control I High Microbial Load (Zymo Research) was used as spike-in. This also applies to DNAour spike-in, but is not the case for *E. coli* as spike-in. *E. coli* was only detected in extractions where 16X *E. coli* was used, which was the highest amount of *E. coli* used.

These results could show the difference in quality of an industry-grade spike-in like the Spike-in Control I High Microbial Load (Zymo Research) in comparison to *E. coli* spike-ins, which were prepared in house. This highlights how important it is to conduct experiments with many controlled variables to obtain reproducible results. Studies using the QIAamp Pathogen UCP kit have demonstrated significantly lower bacterial reads in sequencing data when compared to another kit and its challenges in extracting bacteria DNA when human DNA is also present (Takeuchi et al., 2019). An unexpected bacterium, *Photobacterium leiognathi* was detected by the sequencing results, this could be due to contamination or analysis error. A study has shown that certain DNA extraction contaminants called “Kitome” and “Splashome” could cause false detection in microbiome analysis (Olomu et al. 2020).

4.4 Genes conferring antimicrobial resistance

In this study, only a few genes that could confer antimicrobial resistance were detected, one of the possible reasons why there were few genes conferring antimicrobial resistance identified could be that the DNA was fragmented. This combined with the fact that the EPI2ME software by default only uses coverage and identity values of 80% or more, meaning only reads that cover at least 80% of the gene and have a higher than 80% identity similarity will qualify. Changing the identity value can affect the reliability of the results but changing the coverage value to lower could potentially lead to more hits on the genes conferring antimicrobial resistance properties. This is shown when DNAour controls (*Enterococcus* & *Moraxella*) that were in QDU 28 and DNAour Control had antibiotic resistance gene hits and when the same DNAour (*Enterococcus*) were used in other extractions as spike-in, the results had no hits this time. This could have a few reasons. One is the fact that when used as a spike-in, DNAour spike-ins are going through a second time of DNA extraction, which would affect the DNA integrity and could cause some extra shearing. There is evidence that detecting genes conferring antimicrobial resistance using devices relying on long-read data, becomes challenging when DNA is fragmented during the extraction process (Purushothaman et al., 2024).

4.5 Time & Inclusivity

mNGS offers a broader range of detection of rarer pathogens and certain polymicrobial infections that are more challenging to detect using the traditional methods (Woldeyohannis & Desta, 2024). This study’s sequencing durations were approximately 24 hours, but other studies have used shorter durations thereby reducing the total turnaround time from a day or two to less than a day or even less than half a day (Street et al., 2020). This is an improvement compared to the current gold standard, culturing which could take days for results to come back, and septic patients do not have this amount of time to wait for results.

4.6 Societal, Environmental and Economic significance

The development of new diagnostic methods is important because finding new better methods than those that are currently used could mean better care for patients. Better diagnostics could also mean less false-positive diagnoses that could be a burden on a patient's health, economy and even mental wellbeing. Treatment with antibiotics could contribute to antibiotic resistance, fewer false diagnoses can reduce the misuse of antibiotics and decrease antibiotic resistance (Reyes et al., 2020). This study also explores a better alternative biological fluid, urine, which is considerably easier to collect, and less invasive to the patient in cases with urosepsis. Considering it is also less complicated to store, handle etc. and it requires less resources and reduces inequality as it requires less equipment. This new approach also produces less biohazardous waste compared to current methods such as culturing which involve culturing and cultivating of possibly dangerous pathogens, capable of putting the clinicians at risk and challenging to dispose.

4.7 Ethical considerations

The identity of the participants that donated urine for this experiment will remain anonymous and their donated urine was used with their knowledge and consent. It is also important to consider genders in scientific studies to be inclusive. It is important that methods are used that consider participants well-being and integrity.

This experiment followed all safety measures needed in accordance with risk assessments that were done on the platform KLARA to evaluate and consider all the risks associated with the experiment. This study was conducted with integrity, ethical considerations and accountability in mind.

4.8 Use of Artificial Intelligence

Language model GPT-4o (chatgpt.com) was utilized to aid with coding the python programming that was required for statistics and to summarize some articles key findings.

4.9 Conclusion

An increasing number of studies have been and are in pursuit of early diagnostic methods of bacterial infections to prevent and treat sepsis using urine, blood or plasma to extract or detect biomarkers, DNA and RNA directly instead of culturing bodily fluids and having to wait a long time to get results. Research aiming to personalize infection treatment has combined mNGS and immune response profiling to gain a deeper understanding of the patient's infection (Quarton et al., 2024). Using urine as the biological fluid for research and diagnosis purposes is advantageous because collecting it is less invasive, in addition to it requires less resources to withdraw, store and handle compared to other biological

fluids. The volume of urine used in this study is as low as 1 ml. In this study it was shown that developing better DNA extraction routines and benchmarking DNA extraction kits is as important as the sequencing, and for this method to be reliable and standard, each step of its pipeline needs to be robust and efficient. It is important that future research and development is done using urine samples from suspected UTI patients to test this method and its effectiveness in the field and the real world. Further research with larger datasets and more robust DNA extraction workflows and pipelines are also needed to investigate mNGS and Nanopore Sequencing for their potential as new diagnostic methods.

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Appendix I Python Code

```
import numpy as np
from scipy.stats import shapiro

# Sample data (replace this with your own dataset)
data = []

# Perform Shapiro-Wilk test
stat, p = shapiro(data)

print("Shapiro-Wilk Test:")
print(f"Test Statistic = {stat:.4f}")
print(f"P-value = {p:.4f}")

# Interpretation
alpha = 0.05
if p > alpha:
    print("✅ Data looks normally distributed (fail to reject H0)")
else:
    print("❌ Data does not look normally distributed (reject H0)")
|
```

Figure 2. The statistics python code used for the Shapiro-Wilks analysis

Appendix II Raw Data (QDU)

Table. 10 Raw Data for extractions using the QDU

Extraction Number	Spike (amount of DNA extractions)	Concentration (ng/μl)	260/230	260/280	Elution Volume (μl)
1	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	14.2	1.11	1.76	20
2	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	18.2	1.45	1.68	20
3	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	16.9	1.13	1.71	20
4	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	6.96	1.32	1.60	50
5	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	9.02	1.10	1.67	50
6	16X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	6.48	1.00	1.75	50
7	8X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	8.8	0.61	1.79	50
8	8X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	7.16	1.25	2.66	50
9	8X <i>E. coli</i> + 105 ng DNAour (<i>Enterococcus</i>)	8.84	1.22	1.66	50
10	2X <i>E. coli</i> + 2X Control I	2.54	1.27	1.7	50
11	2X <i>E. coli</i> + 2X Control I	2.46	1.87	1.72	50
12	2X <i>E. coli</i> + 2X Control I	2.18	0.63	1.56	50
13	4X <i>E. coli</i>	4.96	0.65	1.55	50
14	4X <i>E. coli</i>	5.36	1.97	1.7	50

15	4X <i>E. coli</i>	3.6	1.92	1.79	50
16	4X Control I	3.36	0.33	1.87	50
17	4X Control I	2.95	1.25	1.70	50
18	4X Control I	3.32	1.05	1.67	50
19	8X Control I + 105 ng DNAour (<i>Corynebacterium</i>)	2.62	0.28	2.23	50
20	8X Control I + 105 ng DNAour (<i>Corynebacterium</i>)	3.12	0.77	1.65	50
21	8X Control I + 105 ng DNAour (<i>Corynebacterium</i>)	2.18	0.39	1.54	50
22	8X Control I	2.14	0.90	3.16	50
23	8X Control I	1,79	0.82	1.59	50
24	8X Control I	2.90	1.04	-3.73	50
25	*2X Control I	1.21	0.23	1.77	50
26	*2X Control I	1.30	0.55	1.66	50
27	*4X Control I	1.39	0.58	1.88	50
28	*4X Control I	1.47	0.72	1.85	50
29	-	4.3	1.88	4.27	50
30	-	0.892	0.34	2.08	50
31	-	3.44	1.52	2.25	50

*Incubation time at step 7 was 30 minutes.

Appendix III Raw Data (QUP)

Table 11. Each row is one extraction (n=1). If step 1&2 is repeated, urine volume is 2 ml, otherwise 1 ml.

Extraction Number	Spike	Concentration (ng/μl)	260/230	260/280
1	4X Control I (Zymo Research)	1.32	0.21	1.35
2*	4X Control I (Zymo Research)	0.484	0.02	3.62
3	8X Control I (Zymo Research)	0.182	0.06	1.39
4	8X Control I (Zymo Research)	0.134	0.00	-0.88
5	8X Control I (Zymo Research) + 100 ng DNAour (60 ng <i>Haemophilus</i> & 40 ng <i>Corynebacterium</i>)	0.502	0.16	1.58
6	8X Control I (Zymo Research) + 100 ng DNAour (60 ng <i>Haemophilus</i> & 40 ng <i>Corynebacterium</i>)	0.516	0.02	2.35
7	8X Control I (Zymo Research) + 100 ng DNAour (60 ng <i>Haemophilus</i> & 40 ng <i>Corynebacterium</i>)	TOO LOW OUT OF RANGE	0.09	0.82
8	8X Control I (Zymo Research) + 100 ng DNAour (60 ng <i>Haemophilus</i> & 40 ng <i>Corynebacterium</i>)	0.196	0.05	1.72
9	-	0.108	0.10	0.92
10	-	0.194	0.06	1.5

* Step 1 & 2 of the protocol of this kit weren't repeated.