Metagenomic DNA sequencing for semi-quantitative pathogen detection from urine: a prospective, laboratory-based, proof-of-concept study

Victoria A Janes, Sébastien Matamoros, Patrick Munk, Philip T L C Clausen, Sylvie M Koekkoek, Linda A M Koster, Marja E Jakobs, Bob de Wever, Caroline E Visser, Frank M Aarestrup, Ole Lund, Menno D de Jong, Patrick M M Bossuyt, Daniel R Mende, Constance Schultsz

Summary

Background Semi-quantitative bacterial culture is the reference standard to diagnose urinary tract infection, but culture is time-consuming and can be unreliable if patients are receiving antibiotics. Metagenomics could increase diagnostic accuracy and speed by sequencing the microbiota and resistome directly from urine. We aimed to compare metagenomics to culture for semi-quantitative pathogen and resistome detection from urine.

Methods In this proof-of-concept study, we prospectively included consecutive urine samples from a clinical diagnostic laboratory in Amsterdam. Urine samples were screened by DNA concentration, followed by PCR-free metagenomic sequencing of randomly selected samples with a high concentration of DNA (culture positive and negative). A diagnostic index was calculated as the product of DNA concentration and fraction of pathogen reads. We compared results with semi-quantitative culture using area under the receiver operating characteristic curve (AUROC) analyses. We used ResFinder and PointFinder for resistance gene detection and compared results to phenotypic antimicrobial susceptibility testing for six antibiotics commonly used for urinary tract infection treatment: nitrofurantoin, ciprofloxacin, fosfomycin, cotrimoxazole, ceftazidime, and ceftriaxone.

Findings We screened 529 urine samples of which 86 were selected (43 culture positive and 43 culture negative). The AUROC of the DNA concentration-based screening was 0.85 (95% CI 0.81–0.89). At a cutoff value of 6.0 ng/mL, culture positivity was ruled out with a negative predictive value of 91% (95% CI 87–93; 26 of 297 samples), reducing the number of samples requiring sequencing by 56% (297 of 529 samples). The AUROC of the diagnostic index was 0.87 (95% CI 0.79–0.95). A diagnostic index cutoff value of 17.2 yielded a positive predictive value of 93% (95% CI 85–97) and a negative predictive value of 69% (55–80), correcting for a culture-positive prevalence of 66%. Gram-positive pathogens explained eight (89%) of the nine false-negative metagenomic test results. Agreement of phenotypic and genotypic antimicrobial susceptibility testing varied between 71% (22 of 31 samples) and 100% (six of six samples), depending on the antibiotic tested.

Interpretation This study provides proof-of-concept of metagenomic semi-quantitative pathogen and resistome detection for the diagnosis of urinary tract infection. The findings warrant prospective clinical validation of the value of this approach in informing patient management and care.

Funding EU Horizon 2020 Research and Innovation Programme.

Copyright © 2022 The Author(s). Published by Elsevier Ltd. This is an Open Access article under the CC BY 4.0 license.

Introduction

Urinary tract infection is one of the most common reasons for the prescription of antibiotics. It represents the most common nosocomial infection, and the second most common reason for bacteraemia in hospitalised patients.1 The clinical reference standard for detecting urinary tract infection is a semi-quantitative bacterial culture, whereby a fixed volume of urine is streaked onto selective and non-selective agar plates; bacterial colonies of different morphologies are identified and counted after 1–2 days of incubation. This quantification is essential to support or reject a clinical diagnosis of a urinary tract infection, since urinary pathogens can be part of the normal genitourinary microbiota.1 Although urine culture is inexpensive and well standardised, it is relatively slow and potentially biased by antimicrobial treatment received beforehand. Alternative diagnostic tools are desired.

Metagenomic DNA sequencing has been suggested as a diagnostic tool for unbiased and rapid bacterial pathogen detection,2 but practical applications are still rare.3 Proof-of-concept studies have described the use of metagenomic sequencing for sterile-site infections to detect the presence or absence of pathogens.4,5 However, such dichotomous reporting is unsuitable for non-sterile materials, such as urine, in view of the need for quantification.

Hasman and colleagues reported a correlation between bacterial pathogens cultured from urine and those identified by metagenomic sequence analysis.6 This study provides proof-of-concept of metagenomic semi-quantitative pathogen and resistome detection,2 but practical applications are still rare.3 Proof-of-concept studies have described the use of metagenomic sequencing for sterile-site infections to detect the presence or absence of pathogens.4,5 However, such dichotomous reporting is unsuitable for non-sterile materials, such as urine, in view of the need for quantification.6 Hasman and colleagues reported a correlation between bacterial pathogens cultured from urine and those identified by metagenomic sequence analysis.5

Victoria A Janes, Sébastien Matamoros, Patrick Munk, Philip T L C Clausen, Sylvie M Koekkoek, Linda A M Koster, Marja E Jakobs, Bob de Wever, Caroline E Visser, Frank M Aarestrup, Ole Lund, Menno D de Jong, Patrick M M Bossuyt, Daniel R Mende, Constance Schultsz

Lancet Microbe 2022; 3: e588–97
Published Online
June 7, 2022
https://doi.org/10.1016/S2666-5247(22)00088-X
Department of Medical Microbiology (V A Janes MD, S Matamoros PhD, S M Koekkoek BSc, B de Wever, C E Visser MD PhD, Prof M D de Jong MD PhD), D R Mende PhD, Prof C Schultsz MD PhD), Core Facility Genomics, Clinical Genetics (L A M Koster BSc, M E Jakobs MSc), Department of Epidemiology and Data Science (Prof P F M M Bossuyt PhD), and Department of Global Health, Amsterdam Institute for Global Health and Development (AIGHD) (Prof C Schultsz), University of Amsterdam, Amsterdam UMC, Amsterdam, Netherlands; National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark (Prof M Munk, P T L C Clausen, Prof F M Aarestrup PhD, Prof D Lund PhD), Correspondence to: Dr Victoria A Janes, Department of Medical Microbiology, University of Amsterdam, Amsterdam UMC, 1105AZ Amsterdam, Netherlands, v.a.janes@amsterdamumc.nl or Dr Daniel R Mende, Department of Medical Microbiology, University of Amsterdam, Amsterdam UMC, 1105AZ Amsterdam, Netherlands, d.r.mende@amsterdamumc.nl

www.thelancet.com/microbe Vol 3 August 2022 e588

Funding EU Horizon 2020 Research and Innovation Programme.

Copyright © 2022 The Author(s). Published by Elsevier Ltd. This is an Open Access article under the CC BY 4.0 license.
Research in context

Evidence before this study
Metagenomic sequencing is an emerging diagnostic tool used to diagnose bacterial infections; however, clinical studies comparing metagenomics to reference standard diagnostic methods are sparse and focus on sterile-site infections. Urine samples are non-sterile and form up to 40% of the workload in clinical microbiology laboratories. We searched MEDLINE on July 6, 2020 using the terms “metagenomic”, “diagnostic”, “clinical”, “microbiology”, and “bacteria” for studies published in all languages, on humans, between the search date and July 6, 2015. We repeated this search, using the same criteria, on March 28, 2021. Once reviews and case reports were filtered out, our search yielded 45 results, of which seven studied metagenomic diagnostics of bacterial non-sterile site infections. Cross-referencing added a further three studies. Most of these studies reported pathogen presence or absence as a dichotomous outcome; for example, the nanopore-based metagenomics detection of bacterial pathogens from sputum and urine. Dichotomous outcomes are unsuitable for non-sterile site infections, such as urinary tract infections, where bacterial pathogens can be part of the normal microbiota and only cause infection when their abundance reaches above a certain threshold. Moreover, these studies were restricted to selected, heavily infected or spiked, samples, which is not representative of daily diagnostic practice. Only two studies reported pathogen quantification. Cell-free metagenomic sequencing of urine samples from 34 patients undergoing kidney transplantation detected differences in sequence read abundances of pathogens that were indicative of pathogen growth. This method required longitudinal sampling, which is typically not feasible in clinical practice for the diagnosis of an acute infection. A combined pre-sequencing screening approach, in combination with metagenomic sequence analysis, holds promise that the correspondence will be even better when using tools that are developed for similar meta- and pathogen identification.

Methods

Study design and samples
This study was conducted at the Amsterdam UMC (location Academic Medical Center), a tertiary hospital in Amsterdam, the Netherlands. Eligible samples included all urine samples that were collected between Nov 5 and
Dec 31, 2018—according to standard operating procedures of the Academic Medical Center—from patients attending the inpatient or outpatient departments of the hospital, and that were submitted for semi-quantitative culture. Samples were anonymised before inclusion. Exclusion criteria were availability of less than 2 mL of urine or sample storage of more than 7 days.9 Samples were stored at 4°C until processing. Briefly, 2–20 mL of urine was centrifuged (Rotanta 460R, Hettich, Tuttingen, Germany) for 30 s at 2000 g to remove the human cell pellet, followed by centrifugation of the supernatant for 15 min at 4636 g (maximum speed) for pelleting of the remaining (bacterial) cells. The cell pellet was lysed enzymatically (appendix p 2), followed by automated DNA extraction using the NucliSSENS easyMag platform (Biomérieux, Marcy-l’Étoile, France) and DNA quantification using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) at a detection limit of 0·05 ng/µL; all procedures were done according to manufacturer’s instructions.

Samples were processed in batches of 24, including a negative control for detection of cross-contamination and a positive control to monitor DNA extraction efficiency, expressed as ng of DNA per µL of eluate (appendix p 2). Positive extraction controls were not sequenced. Additional controls were included to monitor the sequencing process.

Researchers remained masked to the culture results until DNA extraction was completed and were re-masked for the sample selection and sequence analysis. Researchers had no access to patient data. Results of the diagnostic index were not available to the assessors of the reference standard, but they had full access to electronic patient records.

This study was carried out using anonymised surplus patient materials in accordance with the Amsterdam UMC Research Code and General Data Protection Regulation. Informed consent was waived by the Medical Ethical Committee of the Amsterdam UMC, location Academic Medical Center. The study protocol is available in the appendix (pp 47–54).

Library preparation and internal control
An internal sequencing control consisting of Thermus thermophilus DNA was used as a technical process control.7 Before library preparation, sample DNA was spiked with internal sequencing control at a concentration of 0–5% of the sample DNA concentration. Low DNA concentration samples and negative controls were supplemented with internal sequencing control to meet the minimum requirement for PCR-free library preparation of 50 ng of DNA in 35 µL of eluate.

Unspiked mock community samples (ZymoBIOMICS Microbial Community Standard II; Zymo Research, Irvine, CA, USA) were included in each library preparation batch to estimate the typical amount of cross-contamination and barcode hopping of the internal sequencing control.8 The mean relative abundance of internal sequencing control found in unspiked mock community samples was used to determine the lower threshold of detected internal sequencing control to deem library preparation and sequencing successful.

The KAPA HyperPlus kit (Roche, Basel, Switzerland) was used for PCR-free library preparation according to manufacturer’s specifications (appendix p 2). The Illumina HiSeq4000 platform (Illumina, San Diego, CA, USA) was used to sequence 150 bp paired-end reads at 2 million reads per sample.

Semi-quantitative urine culture and phenotypic antimicrobial susceptibility testing
Urine samples were cultured according to standard operating procedures of the Academic Medical Center clinical microbiology laboratory. Full details are given in the appendix (p 3).

Pre-sequencing screening of DNA concentration
Although a high DNA concentration could suggest a high concentration of bacterial pathogens or a high concentration of bacterial or cellular contaminants, a low DNA concentration indicates a low concentration of any type of bacteria or cells and, therefore, a culture-negative sample. Establishing a cutoff below which culture positivity would be unlikely was thus considered feasible. The total DNA concentration of urine samples was defined as the amount of DNA extracted per mL of centrifuged urine (ng/mL). The area under the receiver operating characteristic curve (AUROC) was calculated using urine culture as the reference standard and DNA concentration as a continuous variable. Criteria for the cutoff value of DNA concentration were defined a priori as a negative predictive value of at least 90% (R-package optimal.cutpoints, version 1.1–4; RStudio, version 1.1.463),11 combined with the highest corresponding test efficiency; the latter was defined as the percentage of culture-negative samples per negative predictive value. Samples with a DNA concentration exceeding the cutoff were randomly selected for sequencing. Full details are given in the appendix (p 46).

Sequence analysis
We used a custom pipeline that calls BBduk2 (version 36.49) from BBTools to remove poor quality reads and adaptors. DNA sequence fragments retaining at least one read after trimming were used in the further analysis.

Bbmap (version 38.35) was used to count and remove human reads (settings: fast, semi-perfect mode, and minimum 1D match 98%).12 Bacterial reads were identified using Kraken2 (version 2.0.8-beta; default settings) and the MiniKraken2_v2.8GB database (downloaded on July 2, 2019).13 The relative abundance of internal sequencing control was calculated as the number of Thermus reads at genus level relative to the total number of reads per sample. All reads mapping to the
genus Thermus were removed before Bayesian re-estimation of reads using Bracken (version 2.2; default settings).14 Pavian (version 0.8.4) was used to remove reads mapping to artificial constructs, Enterobacteria phage PhiX174 sensu latu, and viruses.15 Taxa with fewer than 1000 assigned reads were removed. The remaining species represented 99% of all sequence reads. Bacteria were reported as the relative abundance of all classified bacterial reads.

Bacterial species were annotated as pathogenic or non-pathogenic, based on clinical microbiology practice and a scoping review of the literature (appendix pp 7–14).

**Diagnostic index**

A diagnostic index was designed to provide a semi-quantitative measure to determine a test result as positive or negative. The diagnostic index was defined as the product of the sample’s DNA concentration (ng/mL) and cumulative relative abundance of pathogens (RA\textsubscript{path}) per sample.

\[ \text{DI} = [\text{DNA}] \times (1 – \text{fraction\textsubscript{human}}) \times \sum \text{RA}_\text{path} \]

The sample DNA concentration was corrected for human DNA by multiplication with the fraction of non-human, non-internal sequencing control sequence reads. RA\textsubscript{path} was defined as the sum of the relative abundance of bacterial pathogens, including putative pathogens (appendix pp 7–14). Diagnostic index positivity was defined as a diagnostic index value that was equal to or greater than the cutoff.

The R package (optimal.cutpoints) was used to calculate the AUROC and to identify a cutoff value that maximised the Youden index.11 We corrected the negative and positive predictive values for the culture-positive prevalence of 0.66 among the samples eligible for sequencing.

A false-negative test result was defined as a negative diagnostic index and culture-positive result. A false-positive test result was defined as a positive diagnostic index and culture-negative result. True-positive and true-negative test results were defined as tests with consistently positive or negative results, respectively.

Testing for statistical significance was done with the independent two-group Mann-Whitney U test for non-parametric data and Kendall’s rank correlation for comparing continuous and categorical variables using R (version 4.0.3). p values less than 0.05 were deemed significant.

**Antimicrobial resistance**

Genotypic antimicrobial resistance prediction was compared with phenotypic antimicrobial susceptibility testing for six antibiotics commonly used for urinary tract infection treatment: nitrofurantoin, ciprofloxacin, fosfomycin, cotrimoxazole, ceftazidime, and ceftriaxone.

We used ResFinder (version 4.1) and PointFinder (version 3.0; KMA-1.3.7) tools to identify resistance determinants directly from reads using standard settings, which are designed to annotate antimicrobial resistance facilitated by gene presence and by point mutations, respectively (databases downloaded on Dec 3, 2020).17–19 As part of ResFinder and PointFinder, the identified resistance determinants were matched with an additional genotype-to-phenotype key to produce in-silico antibiograms. The genotype–phenotype agreement per antibiotic was scored as a percentage.

**Statistical analysis**

The sample size was calculated on the basis of the results of a pilot study comprising 41 consecutively collected urine samples (appendix pp 3–4). For pre-sequencing screening of DNA concentration, the desired negative predictive value and positive predictive value (shown as PPV) were at least 90% and at least 30%, respectively. The predefined error margin (EM) of the 95% CI was 5% and the fraction of samples to pass the pre-sequencing screening (frac\_pos) was 0.6. The calculated sample size\textsuperscript{20} was 530:

\[ \text{Sample size} = \frac{1.96^2 \times \text{PPV} \times (1 - \text{PPV})}{\text{EM}^2 \times \text{frac\_pos}} \]

We anticipated that the diagnostic index would distinguish between culture-positive and culture-negative
samples with an AUROC of at least 0·65. To reject the null hypothesis of no discrimination with a type-I error of 5% and a type-II error of 20%, 43 culture-positive and 43 culture-negative samples were needed, as determined via the easyROC web tool.21

A sensitivity analysis—comparing AUROC, negative predictive value, and positive predictive value calculation for the DNA concentration pre-sequencing screening—was performed on all data, including unclassifiable samples deemed to be culture positive.

Role of the funding source
The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
We analysed 619 urine samples, of which 90 were excluded for various reasons, leaving 529 samples with matched culture data available for analysis (figure 1). Of these 529 samples, 158 were culture positive, 351 were culture negative, and 20 were not classifiable.

DNA was at undetectable concentrations in all 27 negative controls except one, in which the concentration was 0·31 ng/µL. Urine samples in this extraction batch had similar DNA concentrations to all other samples (median 1·1 ng/µL [n=22] vs 0·83 ng/µL [n=636]; p=0·68). The DNA concentrations of the positive controls (mean 3·9 ng/µL [SD 1·8]) and mock communities (mean 0·65 ng/µL [0·09]) were normally distributed, supporting the consistency of the DNA extraction method.

26 of 158 culture-positive samples had a DNA concentration below the cutoff and were marked as false-negatives (figure 2A). No quantifiable DNA was measured in 12 of the 26 false negative samples (appendix pp 5, 15). A DNA quantification failure related to a Qubit measurement error was likely to have affected four samples, given that none of the samples quantified in this batch were measured to contain any DNA. A further three of 12 samples grew low concentrations of pathogens (<10⁴ to 10⁴ CFU/mL), whereas no bacteria and no leukocytes were seen in the urine Gram stain, indicating a very low bacterial load. No clear explanation could be identified for the remaining five samples.

We assessed the utility of DNA concentration as a pre-sequencing screening step. DNA concentration distinguished between culture-negative and culture-positive urine samples with an AUROC of 0·86 (95% CI 0·82–0·90). In a sensitivity analysis on all data, in which 20 unclassifiable samples were deemed to be culture positive (figure 1), the AUROC was 0·85 (0·81–0·89; figure 2B). The selected DNA concentration cutoff value of 6·0 ng/mL fit the pre-defined test criteria, yielding a negative predictive value of 91% (95% CI 87–93; 26 of 297 samples), and a positive predictive value of 66% (60–75; 152 of 232). The sensitivity was 85% (78–89; 152 of 178) and the specificity was 77% (72–84; 271 of 351).

This cutoff excluded 297 (56%) of 529 urine samples from sequencing.

The process of library preparation and sequencing was controlled by negative controls (one per batch, 16 total) and spiking samples with internal sequencing control. The mean relative abundance of internal sequencing control and bacteria in negative controls was 96·32% (SD 3·47; 787130 of 811613 reads), and 3·84% (3·53; 24483 of 811613), respectively, indicating minimal contamination.

The minimum detection threshold of internal sequencing control was calculated at 0·72%. All diagnostic index-negative samples, except one, were sequenced successfully. The sample that was not successfully sequenced had an internal sequencing control relative abundance of 0·42%. Nevertheless, the diagnostic index result of 0·3 was in agreement with the culture-negative result.

43 culture-positive and 43 culture-negative urine samples were randomly selected for sequencing. The mean read count per urine sample was 2·1 million (SD 1·0). The number of human reads varied per sample. After the removal of low quality and human reads, a median of 812467 reads (IQR 394646–1378518) per sample remained for bacterial species identification (including Thermus thermophilus reads).

200 bacterial species were identified, of which the most abundant species in culture-positive samples were Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumoniae, Aerococcus urinae, Bifidobacterium longum, Serratia marcescens, Morganella morganii, and Actinomitus schaali (appendix p 6). In culture-positive samples, six species—namely E coli, P aeruginosa, E faecalis, P mirabilis, K pneumoniae, and A urinae—accounted for up to 90% of the total number of sequence reads. By contrast, among the culture-negative samples, 30 species accounted for 90% of the total number of sequence reads, of which the most abundant were Gardnerella vaginalis, E coli, K pneumoniae, Acinetobacter lwoffii, Streptococcus agalactiae, Staphylococcus epidermidis, Bifidobacterium dentium, P aeruginosa, Lactobacillus gasseri, Lactobacillus crispatus, Lactobacillus jensenii, and E faecalis. The median diagnostic index was 34·8 (IQR 18·2–103·7) for culture-positive samples and 4·6 (IQR 2·0–12·0) for culture-negative samples (figure 3A; p=2·5×10⁻⁴, Wilcoxon rank sum exact test). The median diagnostic index after grouping by culture results ranged between 30·4 for samples with 10¹–10⁴ CFU/mL and 96·9 for samples with at least 10⁵ CFU/mL (figure 3B).

The diagnostic index correlated positively with the colony count of cultured pathogens (Kendall’s rank correlation τ 0·48, p=1·7×10⁻⁴; figure 3B). This correlation was stronger than the correlation of the colony count with the individual components of the diagnostic index: DNA concentration (τ 0·35, p=1·3×10⁻²) and cumulative relative abundance of pathogens (τ 0·35, p=9·7×10⁻³).
The AUROC of the diagnostic index was 0.87 (95% CI 0.79–0.95). The calculated optimal cutoff value, based on maximising the Youden index, was 17.2. Assuming a culture-positive prevalence of 66% (152 positive samples out of 232 samples above the pre-sequencing screening cutoff), 39 urine samples were classified as diagnostic index-positive and 47 as diagnostic index-negative—with a positive predictive value of 93% (95% CI 85–97) and a negative predictive value of 69% (55–80; figure 4; appendix pp 16–17). The negative predictive value and positive predictive value of different cutoff values are projected in the appendix (appendix pp 16–17).

Of the 39 diagnostic index-positive samples, 34 were true positives (87%) and, of the 47 diagnostic index-negative samples, 38 were true negatives (81%).

Eight (89%) of the nine false-negative samples grew Gram-positive bacteria, including *S agalactiae*, *Staphylococcus aureus*, or *E faecalis*, in colony counts of about $10^4$–$10^5$ CFU/mL and with diagnostic index values.
of 4·6–13·5. The remaining sample grew more than 10⁵ CFU/mL *Acinetobacter pittii*, but the diagnostic index of this sample was 1·1.

Of the five false-positive samples, three had diagnostic index values of 22–41 and their culture results were interpreted as faecal contamination. The remaining two samples showed no growth in culture, but had diagnostic index values of 92 and 55, respectively. Both samples contained few classified bacterial reads (29,477 and 48,643, respectively), comprising relative abundances of 78% (23,164 of 29,477) *E coli* and a cumulative relative abundance of 60% (13,368 of 22,050) *P aeruginosa*, *E coli*, and *A lwoffi*.

The genotype–phenotype agreement for the tested antibiotics varied between 71% (22 of 31 samples) and 100% (six of six). Metagenomic sequence analysis correctly predicted the phenotype of all six samples containing extended-spectrum β-lactamase producing *E coli*. The remaining non-extended-spectrum β-lactamase Enterobacteriaceae showed 71% (22 of 31) correspondence between phenotype and metagenomics prediction for the tested third-generation cephalosporins. The values for genotype–phenotype agreement were 92% (35 of 38) for ciprofloxacin, 72% (13 of 18) for fosfomycin, 76% (29 of 38) for cotrimoxazole, and 88% (30 of 34) for nitrofurantoin. Four samples containing mixed growth (MS_0058, MS_0151, MS_0724, and MS_0730) demonstrated phenotypic nitrofurantoin resistance in culture, but not by metagenomics (figure 5). None of these samples contained *P mirabilis*, which is intrinsically resistant to nitrofurantoin. No resistance determinants were detected for sample MS_1312 (figure 5), although this sample was phenotypically resistant to all tested antibiotics. The cultured bacteria *E coli* and *P mirabilis* only formed 0·48% (12,346 of 2,568,056) of the total number of sequenced reads.

### Discussion

This study provides proof-of-concept of a semi-quantitative metagenomic sequencing approach for urinary tract infection diagnostics, in a clinical microbiology setting. We used a straightforward algorithm based on sample bacterial DNA concentration and the cumulative relative abundance of pathogens to compare test characteristics to urinary culture (the clinical reference standard).

In 271 (77%) of 351 culture-negative urine samples, culture positivity could be excluded with high probability, based on DNA concentration only, within about 1 h—ie, the time needed for sample centrifugation, DNA extraction, and quantification. This screening step could reduce the number of samples requiring sequencing by 56%, potentially leading to substantial cost reductions.

Metagenomics is reported to be a more sensitive bacterial pathogen detection tool than culture.2,3,22 Our study is in agreement with these findings. Inclusion of a bead-beating step before DNA extraction could have improved the lysis and, therefore, the detection of Gram-positive microorganisms. Indeed, eight of the nine false-negative samples contained Gram-positive bacteria and results of the mock community also suggested better detection of Gram-negative than Gram-positive bacteria (appendix p 17). However, in all other samples, metagenomics identified more species than urine culture did, including urinary pathogens that are often missed in culture, such as *A schaalii* (appendix p 6). This finding might have several explanations. The 2–20 mL of urine used for metagenomics might better capture the sample microbiota, leading to more sensitive pathogen detection than the 10 µL of urine used for semi-quantitative culture. Furthermore, the current practice of urine culture is restricted in its ability to grow microorganisms that
require more complex growth conditions than are typically used in routine diagnostic practice. As such, the definition of urinary pathogen might change over time with enhanced diagnostic tools, including metagenomic sequencing. In addition, antibiotic usage before urine sampling might sterilise cultures, but metagenomics could still detect pathogen DNA.

This study has a few limitations. A limitation of the diagnostic index was the interpretation of test results for samples that exhibited abundant growth of multiple pathogens. Three urine samples had diagnostic index values of 22–41 and were, thus, marked as diagnostic index-positive, but the culture results were reported as faecal contamination. These results show that this proof-of-concept study now warrants a prospective clinical study in which the performance of the diagnostic index to support a clinical diagnosis of urinary tract infection is assessed. Such a study should include clinical details—such as signs and symptoms, the sample collection method, and the presence or absence of a urinary

Figure 5: Heatmaps of phenotypic antimicrobial susceptibility and genotypic antimicrobial resistance prediction
Phenotypic antimicrobial susceptibility testing results for six antibiotics (A) and genotypic antimicrobial resistance prediction (B). ResFinder 4.1 and PointFinder 3.0 databases were used, which annotate antimicrobial resistance facilitated by gene presence and by point mutations, respectively. Genotypic antimicrobial resistance prediction was detected with three levels of certainty, depending on the percent identity match of the antimicrobial resistance conferring genetic element with the reference gene, and coverage of the reference gene.25 Sample names and the cultured pathogens are indicated. Resistance determinants found in ResFinder are indicated with an R after the antibiotic name, and PointFinder with a p.*Cotrimoxazole is a combination of trimethoprim and sulfamethoxazole.
catheter—and follow-up of patients after treatment for urinary tract infection, to ensure the appropriate interpretation of diagnostic index results for diagnosing urinary tract infection. A clinical study would also allow us to assess whether metagenomic sequencing can improve the diagnostic accuracy for urinary tract infection over the gold standard of urine culture, which this proof-of-concept study does not allow. Finally, a clinical study would further clarify the minimum infrastructure, as well as logistics and time required, for metagenomics-based diagnostics in the clinical laboratory.

Current knowledge of the composition of the healthy urinary microbiota and the changes that occur during urinary tract infection suggest that the presence of Lactobacillus species is associated with a healthy bladder microbiota, whereas bladder catheterisation—which is common in hospitalised patients—promotes a microbiota profile dominated by G vaginalis, which is associated with recurrent urinary tract infection and might facilitate E coli cystitis. G vaginalis was frequently detected in both culture-positive and culture-negative samples. By contrast, only three of the 30 most common species identified in culture-negative samples were different Lactobacillus spp (L gasseri, L crispatus, and L jensenii). These findings might be reflective of the hospitalised population of this study. More insight is needed into the dynamics of the healthy and diseased urinary microbiota. Although the current approach requires a (putative) pathogen to be defined, future metagenomic diagnostic procedures could expand to uncover new pathogens and consider the overall composition and diversity of the urinary microbiota, including yeast and fungi (which were not considered here), in the diagnosis of urinary tract infection. Finally, our approach might also be evaluated for the diagnosis of other non-sterile site infections. For example, the composition of the lung microbiota has been shown to be relevant for the distinguishment of pathogen colonisation from infection and the outcomes of patients who are critically ill. By contrast, the high bacterial load of the intestinal microbiota is likely to preclude the application of the diagnostic index for diagnosis of, for example, enteric infections.

Currently, the performance of metagenomic antimicrobial resistance prediction is too variable to guide antimicrobial treatment of patients. However, the very good prediction of ciprofloxacin and extended-spectrum β-lactamase isolate susceptibility reported in this study is promising. The polymicrobial composition of the urinary microbiota, and the presence of human DNA, complicates genotypic antimicrobial resistance prediction—as was seen for one sample (figure 5, MS_1312) where the cultured bacteria E coli and P mirabilis only formed 0.48% (12 346 of 2 568 056) of the total number of sequenced reads, rendering the sequence depth too low for detection of resistance genes. Current tools for the detection of antimicrobial resistance genes are designed for whole-genome sequencing, where the expected genome coverage is much higher than for metagenomic sequencing of clinical samples. Relaxed settings for genome coverage and percent identity match might aid antimicrobial resistance gene detection from metagenomic datasets, but simultaneously might lead to spurious detection of antimicrobial resistance genes from minority bacteria that hold no clinical relevance. Machine learning approaches are currently under development and might prove useful for establishing optimal cutoffs for antimicrobial resistance gene coverage and percentage identity match. In summary, this study provides proof-of-concept of metagenomic semi-quantitative pathogen detection for the diagnosis of urinary tract infection, including resistome prediction. A prospective patient-based evaluation of our metagenomic diagnostic approach clearly is warranted to assess its test characteristics in the clinical diagnosis of urinary tract infection.

Contributors
VAJ, SM, BdW, MDdJ, PMMB, and CS conceptualised the study. SM, PM, PTLCC, MEJ, CEV, OL, PMMB, DRM, and CS supervised the study. VAJ, SMK, and LAMK did the investigation work. PM and PTLCC did the software. VAJ and PM curated the data. VAJ and DRM analysed the data. VAJ, PM, PMMB, and DRM led data visualisation. VAJ, SM, DRM, and CS accessed and validated the underlying data. MDdJ and CS provided funding. CEV, FMA, OL, Mdd, and CS provided resources. VAJ wrote the original draft of the manuscript; all other authors reviewed and edited the manuscript.

Declaration of interests
We declare no competing interests.

Data sharing
The metagenomic datasets are publicly available at the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under project accession number PRJEB45363. All metadata are available in the appendix (pp 18–45).

Acknowledgments
This study was funded by the EU’s Horizon 2020 Research and Innovation Programme under grant agreement number 643476 (COMPARE).

References
1. Wilson ML, Gaido L. Laboratory diagnosis of urinary tract infections in adult patients. Clin Infect Dis 2004; 38: 1150–58.
2. Schlaberg R, Chiu CY, Miller S, Procop GW, Weinstock G. Validation of metagenomic next-generation sequencing tests for universal pathogen detection. Arch Pathol Lab Med 2017; 141: 776–86.
3. Wilson MR, Sample HA, Zorn KC, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. N Engl J Med 2019; 380: 2327–40.
4. Ivy MI, Thoenel MJ, Jeraldo PR, et al. Direct detection and identification of prostatic joint infection pathogens in synovial fluid by metagenomic shotgun sequencing. J Clin Microbiol 2018; 56: e00402–18.
5. Hasman H, Saputra D, Sicherritz-Ponten T, et al. Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. J Clin Microbiol 2014; 52: 139–46.
6. Schmidt K, Mwaiguwisa S, Crossman LC, et al. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. J Antimicrob Chemother 2017; 72: 104–14.
7 Charalampous T, Kay GL, Richardson H, et al. Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. *Nat Biotechnol* 2019; 37: 783–92.
8 Ng HH, Ang HC, Hoe SY, et al. Simple DNA extraction of urine samples: effects of storage temperature and storage time. *Forensic Sci Int* 2018; 287: 36–39.
9 Janes VA, van der Laan JS, Matamoros S, Mende DR, de Jong MD, Schultz C. *Thermus thermophilus* DNA can be used as internal control for process monitoring of clinical metagenomic next-generation sequencing of urine samples. *J Microbiol Methods* 2020; 176: 106005.
10 Minich JJ, Sanders JG, Humphrey G, Gilbert JA, Knight R. Quantifying and understanding well-to-well contamination in microbiome research. *mSystems* 2019; 4: e00186–19.
11 López-Ratón M, Rodríguez-Álvarez MX, Cadarso-Suárez C, Gude-Sampedro F. OptimalCutpoints: an R package for selecting optimal cutpoints in diagnostic tests. *J Stat Softw* 2014; 61: 1–36.
12 Bushnell B. BBMap: a fast, accurate, splice-aware aligner. 9th Annual Genomics of Energy & Environment Meeting; March 17–20, 2014 (report e1241166).
13 Lu J, Breitbart FP, Thielen P, Salzberg SL. Bracken: estimating species abundance in metagenomics data. *PeerJ Comput Sci* 2017; 3: e104.
14 Breitbart FP, Salzberg SL. PaviaN: interactive analysis of metagenomics data for microbiome studies and pathogen identification. *Bioinformatics* 2020; 36: 1303–04.
15 Youden WJ. Index for rating diagnostic tests. *Cancer* 1950; 3: 32–35.
16 Vinnikstaya V, Kaas RS, Ruppe E, et al. ResFinder 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* 2020; 75: 3491–500.
17 Zankari E, Allesøe R, Joensen KG, Cavaco LM, Lund O, Aarestrup FM. PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *J Antimicrob Chemother* 2017; 72: 2764–68.