Brief Report

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Usefulness of next-generation DNA sequencing for the diagnosis of urinary tract infection

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SUMMARY Acute urinary tract infection (UTI) is a highly common clinical condition. Although bacterial culture is the gold standard diagnostic test, false negative results may be possible, leading to the pathogen being unidentified. In recent years, bacterial DNA sequencing analysis has garnered much attention, but clinical studies are rare in Japan. In this study, we assessed the usefulness of next-generation DNA sequencing (NGS) analysis for acute UTI patients. We thus performed an observational, retrospective case series study. Urine and blood samples were collected from ten acute UTI patients, of whom four had also been diagnosed with urosepsis. Seven variable regions of bacterial 16S rRNA genes were amplified by PCR and then sequenced by IonPGM. The identified bacterial species were compared with those identified using the culture tests and the clinical parameters were analyzed. As a result, the NGS method effectively identified predominant culture-positive bacteria in urine samples. The urine NGS also detected several culture-negative species, which have been reported to be potentially pathogenic. Out of four urosepsis cases, three were pathogen-positive in blood NGS results, while two were pathogen-negative in blood culture. In one sepsis case, although blood culture was negative for *Escherichia coli*, this species was detected by blood NGS. For non-sepsis cases, however, blood NGS, as well as blood culture, was less effective in detecting bacterial signals. In conclusion, NGS is potentially useful for identifying pathogenic bacteria in urine from acute UTI patients but is less applicable in patients who do not meet clinical criteria for sepsis.

Keywords Next-generation DNA sequencing, 16S rRNA amplicon sequencing analysis, urinary tract infection

1. Introduction

Urinary tract infection (UTI) is a highly common condition diagnosed at emergency departments (ED) worldwide. The pathology of UTIs has various subdivisions, notably upper or lower, simple or complex, and minor or serious (*1*). Urosepsis has high morbidity and mortality rates, and as such, its early diagnosis and treatment are essential (*2*). Clinicians diagnose patients with UTIs comprehensively based on clinical conditions and their clinical parameters, collect urine and blood samples, and begin broad-spectrum antibiotic treatment experimentally (*3*). Later, when the UTI diagnosis is more definite based on the culture results, more targeted antibiotics are prescribed. However, if the culture test is negative, the pathogenic bacteria remains unidentified. This is potentially due to previous antibiotic exposure, error at the time of sampling, an insufficient sample volume, and/or unculturable bacteria (*4*). Unidentified pathogenic bacteria can cause many issues in the quality of diagnosis and treatment, such as the administration of less-effective antibiotics, and futile repeated antibiotic administration. These situations can escalate medical costs.

While the gold standard test for pathogenic bacteria is still the conventional culture test, bacterial DNA sequencing analysis tests are gaining attention in recent years (*5,6*). This is due to recent advances in next-generation DNA sequencing (NGS) technology. Nevertheless, there is a lack of clinical studies comparing conventional culture tests and bacterial DNA sequencing analysis, as well as medical case data. Furthermore, blood cultures in addition to urine cultures are recommended for complex pyelonephritis and urosepsis (*7*). Identifying the pathogenic bacteria in bacteremia patients is very important owing to its

potential impact on patient condition. However, few studies have examined both urine and blood samples by comparing cultures and NGS, and included clinical contexts. Here we investigated the advantages and limitations of using NGS as a method to identify pathogenic bacteria in ED patients with acute UTI. To our knowledge, this is the first report addressing these issues in Japan.

2. Materials and Methods

2.1. Definition of UTI

UTIs were diagnosed comprehensively through a combination of clinical condition, urinalysis, and imaging test findings after excluding other sources of infection. Acute cystitis included typical urinary symptoms such as urinary urgency, frequent urination, painful urination, abdominal pain, and suprapubic pain. Acute pyelonephritis included chills, fever, flank pain, costovertebral angle tenderness, nausea, and vomiting. Urosepsis and septic shock were also included, and diagnoses followed the definition of sepsis (sepsis-3) (*8*). Urinalysis included qualitative examination of urine sediment, white blood cell count (400 × magnification), nitrous acid level, Gram staining, and culture bacterial count. Non-contrast CT scans assessed hydronephrosis, ureteropelvic enlargement, ureteral calculus, and gas formation. Contrast CT scans were used to search for renal abscess and localized sparse pathological changes.

2.2. Ethics and study design

Here we performed an observational and retrospective case series study, which was approved by the Clinical Ethical Committee of Tokai University Medical School (14R220). This study also conforms to the Helsinki Declaration. We conducted this study at two hospitals of Tokai University in Japan between January 2017 and December 2017. The study subject group consisted of ED cases of acute UTI, including subgroups such as upper and lower, simple and complex, and mild and severe. Samples were only collected after explaining the details of the study to the patients and receiving their written consent or that of their families. A total of ten patients agreed to participate in the study and their urine and blood samples were collected in sterile containers and heparin-coated evacuated blood collection tubes, respectively, by experienced registered nurses or physicians operating in a standard disinfected and hygienic manner. Unless otherwise noted, blood and urine samples were collected before the initial administration of antibiotics and subjected to culture and NGS tests. All patients underwent antibiotic treatment based on conventional urine and blood culture. The results of the urine and blood NGS tests were not revealed to patients or to their attending physicians, and thus these results did not influence their treatment. For all cases, after the patients were discharged from the hospital, a retrospective examination was conducted comparing the NGS and culture test results, taking into account the clinical contexts.

2.3. NGS Methodology (Bacterial 16S rRNA amplicon sequencing analysis)

Blood and urine samples were aliquoted and stored at -80°C until use. DNA in urine was isolated using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) with some modifications to the manufacture's protocol. Urine (~1 mL) was thawed and centrifuged at 10,000 × g for 10 min, and particulate materials were collected. The particulates were suspended in 180 µL of a lysis buffer (1.2% Triton X-100, 20 mg/mL lysozyme, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0)) and incubated at 37°C for 30 min. Next, 4 µL of RNase (100 mg/mL) was added and the reaction was allowed to stand for 2 min at room temperature (22-25°C). Proteinase K (25 µL) and buffer AL (200 µL), both supplied with the kit, were then added to the mixture, followed by incubation at 56°C for 30 min. After mixing with 200 µL ethanol, the mixture was applied to the column. Subsequent purification processes were carried out according to the manufacturer's instructions.

Bacterial DNA in blood was isolated as described previously (*9*). Bacterial 16S rRNA gene variable regions were amplified from both DNA samples and were sequenced by IonPGM followed by our in-house metagenomic data analysis pipeline, Genome Search Toolkit (GSTK, *http://kirill-kryukov.com/study/tools/gstk/*) with 5840 representative bacterial species' genomes (*http://genomesync.org*) as described previously (*9*).

In this study, we assumed that a bacterial species exists in a given sample if the number of sequence reads corresponding to the species exceed 10% of those corresponding to all bacterial species.

3. Results and Discussion

3.1. Characteristics of patients with acute UTI

A total of ten acute UTI patients (three male and seven female, median age: 85 years) were included in this study. The patients' characteristics are summarized in Table 1. Of the ten cases, two were lower- and eight were upper-UTIs. Three were uncomplicated and seven were complicated. Cases 1-6 were diagnosed as non-sepsis and cases 7-10 as urosepsis. The range of sequential organ failure assessment (SOFA) scores among the urosepsis cases was 3-17, indicating mild to severe organ failure. Initial antibiotics were selected

Table 1 Characteristics of 10 patients with acute urinary truct infection

Case	Age/sex	Onset of UTI	Type of UTI (cause)	Sepsis(SOFA)	Septic shock	Initial laboratory data	Initial Antibiotics	Definitive Antibiotics	ICU stay	Hospital stay	Outcome
1	89, M	Community	Complicated acute cystitis (prostate cancer)	No	No	WBC 9300/µL CRP 1 mg/dL	ABPC/SBT	Same	No	34 days	Improved
2	89, F	Community	Uncomplicated acute cystitis	No	No	WBC 17800/µL CRP 16 mg/dL	ABPC/SBT	CTRX	No	14 days	Improved
3	86, F	Community	Uncomplicated acute pyelonephritis	No	No	WBC 9300/µL CRP 3 mg/dL	CTRX	Same	No	12 days	Improved
4	74, F	Medical facility	Complicated UTI (neurogenic bladder)	No	No	WBC 13600/µL CRP 7 mg/dL	CTRX	Same	No	1 days	Unknown
5	68, F	Community	Complicated UTI (ureteral calculi)	No	No	WBC 13600/µL CRP 51 mg/dL	LVFX	Same	No	18 days	Improved
6	84, M	Medical facility	Complicated acute pyelonephritis (neurogenic bladder)	No	No	WBC 8300/µL CRP 21 mg/dL	CTRX	Same	No	6 days	Improved
7	86, F	Medical facility	Uncomplicated acute pyelonephritis	Yes (3)	No	WBC 9300/µL CRP 4 mg/dL	CTRX	Same	No	34 days	Improved
8	66, F	Community	Complicated UTI (ureteral calculi)	Yes (8)	Yes	WBC 24300/µL CRP 22 mg/dL	CFPM TOB VCM	CTRX	3 days	11 days	Improved
9	86, M	Medical facility	Complicated UTI (renal abscess)	Yes (11)	Yes	WBC 30200/µL CRP 12 mg/dL	CFPM CPFX VCM	CTRX	10 days	95 days	Improved
10	82, F	Community	Complicated UTI (ureteral calculi)	Yes (17)	Yes	WBC 27200/µL CRP 46 mg/dL	CTRX VCM	Same	2 days	2 days	Death

ABPC/SBT, Ampicillin/sulbactam; CFPM, Cefepime; CPFX, Ciprofloxacin; CRP, C reactive protein (normal < 0.3 mg/dL); CTRX, Ceftriaxone; ICU, intensive care unit; LVFX, Levofloxacin; SOFA, sequential organ failure assessment; TOB, Tobramycin; UTI, urinary tract infection; VCM, Vancomycin; WBC, white blood cell.

empirically with reference to the clinical background, severity, and Gram stain test results. Three patients were admitted to the intensive care unit. Nine patients improved, but one died.

3.2. Comparison of culture and NGS results

Table 2 summarizes our comparison of culture and NGS results from the patients' urine and blood samples. Nine out of ten cases were pathogen-positive in urine culture results, whereas all ten were pathogen- positive in urine NGS results. In most cases, the pathogenic species identified in the urine culture were also predominant in the urine NGS data. However, urinary NGS detected many additional bacteria that were negative in urine culture. Two out of the four urosepsis cases (Case 7-10) were pathogen- positive in blood culture results, and three were pathogen-positive in blood NGS results. In case 7, only contamination was detectable, in culture and NGS analysis of blood. In non-sepsis cases (Cases 1-6), blood culture and NGS testing detected some bacteria that were different from those detected by culture and NGS analysis of urine.

3.3. Usefulness of NGS for the diagnosis of acute UTI

In this study, we evaluated an NGS analysis of 16S rRNA gene amplicons as a new method of detecting pathogenic bacteria in acute UTI patients by studying a group of cases. The study revealed the advantages and limitations of NGS testing in comparison with conventional culture testing of urine and blood. The following three points were demonstrated as clinically important for utilizing NGS testing. Firstly, our results imply that urine NGS testing is not only a potential substitute for urine culture testing but is also useful for detecting other dangerous unculturable bacteria. Secondly, blood NGS testing may be useful for detecting severely spreading pathogenic bacteria in severe urosepsis cases. Lastly, it seems likely that our data from non-severe, non-sepsis cases reflect the potential of NGS for detecting false-positive bacteria due to contaminants or noise, as a result of low abundance of infecting bacteria in the blood.

3.4. Advantages of urine NGS in acute UTI patients

In support of the usefulness of urine NGS testing, we found that in most cases, urine culture-positive pathogenic bacteria showed the highest occupancy rates in urine NGS. Furthermore, urine NGS detected many additional bacteria that were negative in the urine culture. As discussed below, we found some evidence supporting the contention that this result reflected more sensitive detection of pathogenic bacteria by urine NGS, rather than contamination.

The pathogenic bacteria detected in urine culture NGS tests are shown in Figure 1. In this study, the pathogenic bacteria detected in both the urine culture and NGS tests were *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, Enterococcus faecalis, and *Aerococcus urinae*, which are all common UTI pathogenic bacteria (*10,11*). In addition, many other bacteria detected in the urine NGS tests were negative in the urine culture tests. The NGS-detectable, unculturable bacteria included some pathogenic bacteria occasionally found in UTIs by previous studies (*10-14*). Possibly owing to prior antibiotic administration, Case 6 tested negative for *Aerococcus urinae* in a urine culture test, but positive in a urine NGS test. This could have been the causative bacteria, whose DNA was detected by NGS from its dead remnants. Therefore, urine NGS tests may be able to detect bacteria even after antibiotic administration (*15*). For Case 7, the urine culture test showed *Streptococcus species*, but the urine NGS test showed *Aerococcus urinae*, which previous evidence suggests is likely to be the true causative bacterium. *Aerococcus urinae* are often difficult to isolate by urine culture and are therefore suitable for diagnosis by genome analysis (*16*).

A recent study comparing urine culture and NGS testing reported that in acute cystitis cases, urine NGS testing demonstrates good diagnostic performance and is helpful in medical treatment (*17*). However, this study was limited to acute cystitis cases, and did not examine detailed information about the pathogenic bacteria detected, nor did it perform blood NGS tests simultaneously.

3.5. Advantages of blood NGS in urosepsis patients

Our second main finding was evidence supporting the usefulness of blood NGS testing for detecting pathogenic bacteria in very severe cases of urosepsis. Two out of four urosepsis cases (Cases 7-10) were pathogen-positive in blood culture results, and three were pathogen-positive in blood NGS results. Therefore, blood NGS may be able to detect pathogenic bacteria more sensitively than (or at a comparable level to) blood culture.

Figure 2 shows the occupation ratios of bacteria based on urine and blood NGS tests of urosepsis cases. Notably, the blood culture test of Case8 was negative for *Escherichia coli*, but this species was detected in both blood and urine by the NGS tests. *Escherichia coli* was also detected in both blood culture and blood NGS tests in Case 10, coinciding with the results of the upper urinary tract culture from ureteral calculus. Ureteral calculus UTIs are highly likely affected by pathogenic bacteria from the upper area rather than the obstruction area, and the bacteria can differ from those in the urine of the lower area (*18*). In urosepsis cases, the causative bacterium commonly detected by both blood culture tests and blood NGS tests was *Escherichia*

Table 2 Comparison of urine culture and urine NGS, blood culture and blood NGS

Case	Antibiotics before sampling	Urine culture (colony forming units/mL)	Urine NGS	Blood culture	Blood NGS
1	No	*Enterococcus faecalis* (10^4 cfu/mL)	*Enterococcus faecalis* 49%	Negative	No bacteria (more than 10%)
2	No	*Proteus mirabilis* (10^7 cfu/mL) *Lactobacillus species* (10^7 cfu/mL)	*Proteus mirabilis* 58% *Actinotignum schaalii* 14%	Negative	*Bacteroides vulgatus* 16%
3	No	*Klebsiella pneumoniae* (10^6 cfu/mL)	*Klebsiella pneumoniae* 18% *Salmonella enterica* 13% *Enterobacter lignolyticus* 10%	Negative	No bacteria (more than 10%)
4	Yes	*ESBL Escherichia coli* (10^6 cfu/mL)	*Escherichia coli* 65%	Negative	No bacteria (more than 10%)
5	No	*Escherichia coli* (10^6 cfu/mL)	*Escherichia coli* 55%	*Staphylococcus epidermidis*	*Staphylococcus capitis* 20% *Staphylococcus epidermidis* 18% *Comamonas kerstersii* 12% *Corynebacterium aurimucosum* 10%
6	Yes	Negative	*Aerococcus urinae* 20%	*Bacillus species*	No bacteria (more than 10%)
7	No	*Streptococcus species* (10^7 cfu/mL) *Escherichia coli* (10^6 cfu/mL)	*Aerococcus urinae* 51% *Escherichia coli* 19%	*Staphylococcus epidermidis*	*Corynebacterium glucuronolyticum* 12%
8	No	*Escherichia coli* (10^2 cfu/mL)	*Escherichia coli* 57%	Negative	*Escherichia coli* 28%
9	No	*Escherichia coli* (10^7 cfu/mL)	*Escherichia coli* 48% *Streptococcus dysgalactiae* 14%	*Escherichia coli*	*Escherichia coli* 52%
10	No	*Lower urine *Aerococcus urinae* (10^5 cfu/mL) *Lactobacillus species* (10^5 cfu/mL) *Enterobacteria* (10^3 cfu/mL) *Upper urine *Escherichia coli* (10^6 cfu/mL)	*Lower urine *Aerococcus urinae* 25% *Lactobacillus gasseri* 14% *Escherichia coli* 12% *Upper urine Not tested	*Escherichia coli*	*Escherichia coli* 11%

coli, which was considered to be the likely causative bacteria because it is the most common pathogenic bacterium in urosepsis (*19*). Blood NGS testing is therefore useful for identifying the pathogenic bacteria that are clinically most important and influential. This requirement is also present for blood culture testing in febrile UTIs (*20*).

A recent study of ICU patients with suspected sepsis reported that, in comparison with blood culture testing, blood NGS testing had the advantage of detecting multiple pathogenic bacteria. Furthermore, diagnostic sensitivity was significantly higher in blood NGS than blood culture (*21*).

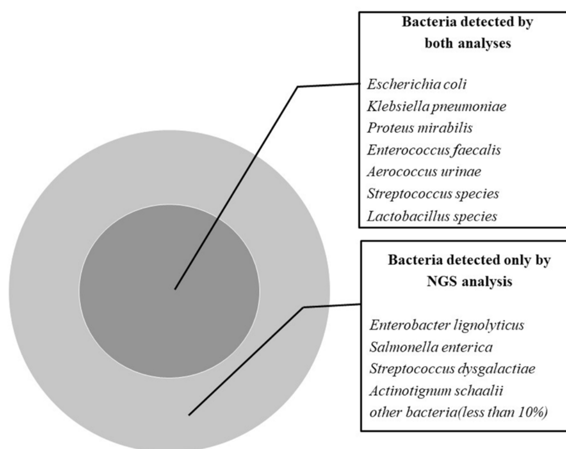

Figure 1. Venn diagram of urine culture and urine NGS results for all UTI cases. The pathogenic bacteria detected in both the urine culture and NGS tests were *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Aerococcus urinae*, which are all common UTI pathogenic bacteria. In addition, many other bacteria detected in the urine NGS tests were negative in the urine culture tests.

3.6. Disadvantages of blood NGS in non-sepsis patients

Our third major finding was that, in less-severe, non-sepsis UTI cases (Cases 1-6), blood NGS testing requires increased caution regarding the possibility of false-positives due to contamination. In Cases 1, 3, 4, and 6, although no bacterial species exceeded the threshold occupancy rate of 10% in the blood NGS test, a large number of bacterial species with extremely low occupancy rates were detected. In Case 5, some bacteria with occupancy rates over 10% were detected with the blood NGS test, but the bacteria detected by the urine and blood NGS tests were different, suggesting that they may have been false-positives. Generally, in UTI-induced bacteremia, the same bacterial species are detected in urine and blood (*22*). Previous studies have addressed the question of whether bacteria cultured from blood are true bloodstream infections or contaminant bacteria. In light of these reports, we suspect that the blood NGS results of Case 5 may represent contaminations (*23*).

The primary cause of false positives is live bacterial contamination, but another cause is DNA contamination from dead bacteria after sterilization. It is difficult to completely remove contamination, and its most frequent source is the patient's skin at the time of drawing blood (*24*). Furthermore, because NGS testing involves several steps, contamination can occur from the environment or the experimental materials used in testing (*21*). The second cause of false positives is related to the fact that NGS testing displays the relative abundance of bacteria, rather than their absolute quantities. That is, even if no pathogenic bacteria are present in the blood from healthy donors, the blood NGS test inevitably amplifies small quantities of contaminants.

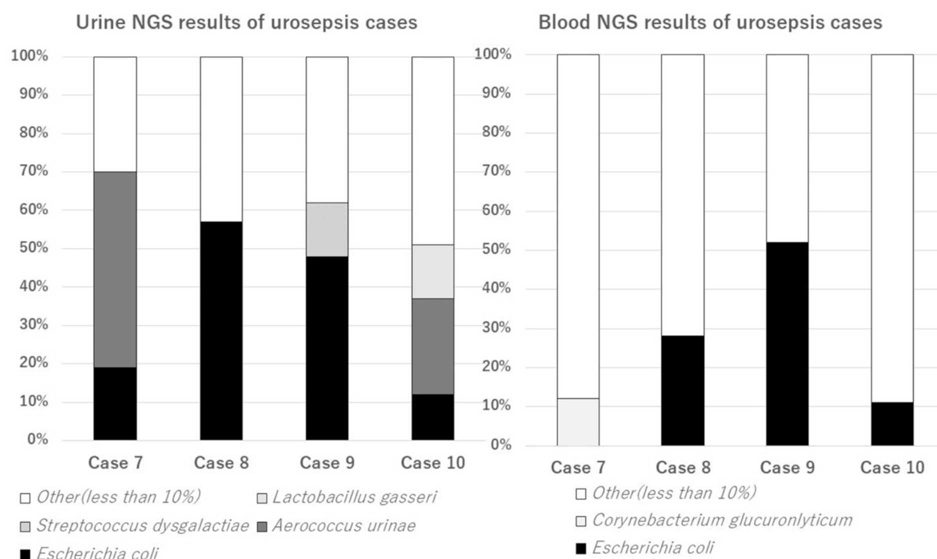

Figure 2. Bacterial occupancy rate based on urine and blood NGS in urosepsis cases. In cases 8-10, both urine and blood NGS results are positive for *Escherichia coli*, which is the most important causative bacteria of urosepsis cases.

Furthermore, a recent study reported that many anaerobic bacteria can be detected in the blood of healthy people by NGS, suggesting the presence of asymptomatic bacteremia (*25*). The detection of bacteria with little or no pathogenicity in the blood of healthy people can cause confusion in clinical diagnosis and treatment. Thus, the results of blood NGS tests for low-severity non-sepsis UTI cases are not reliable.

We propose the following two practices to overcome these limitations of blood NGS testing. First, NGS tests should be conducted on both urine and blood. We also suggest conducting separate NGS tests of blood taken from two or more different sites, before any antibiotics are administered to the patient. This would be useful for distinguishing true infection from contamination and is a standard method in blood culture tests (*26*). The second proposal is to use blood NGS testing for cases that are highly likely to be bacteremia. As predictors for true bacteremia, fevers and increased white blood cell counts are not sufficient; it has been reported that shaking chills are a more useful indicator (*27*). Additionally, there is a strong relationship between having quick SOFA ≥ 2 and bacteremia (*28*). In fact, this study also used quick SOFA ≥ 2 as a criterion of diagnosing urosepsis, in line with the guideline for sepsis-3.

3.7. Limitations of this study

This study has several notable limitations. Firstly, it was confined to retrospective observational analysis of a group of cases, and no statistical analysis was carried out. Furthermore, before the practical application of NGS testing, practical limitations including turnaround time and cost have to be addressed. It takes nearly one week to complete the entire process, from preparing the library, to using the GSTK program, and to analyzing data. For us, the material cost of an NGS test is around $100 (USD). To use NGS testing practically, these issues will need to be overcome (*9*).

4. Conclusion

The results of this study suggest that as a method of identifying pathogenic bacteria in acute UTI patients, urine NGS testing is highly accurate and useful. For sepsis patients, conducting blood NGS testing in parallel may increase the likelihood of detecting the most clinically important pathogenic bacteria. On the other hand, NGS testing involves some limitations that need to be overcome, such as the need to optimize the criteria for applying this method, the qualitative nature of the results, contamination, and the time and expense of the tests. Nevertheless, for cases in which the pathogenic bacteria are unculturable, this method deserves attention as a new and highly sensitive diagnostic technique. To refine this method, it will be important to accumulate more clinical data.

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