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Tracking of *Klebsiella pneumoniae* and *Klebsiella aerogenes* in Urinary Tract Infection Cases among Hemodialysis patients by Conventional and Advanced Molecular Methods

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Abstract:

Background: Bacteria cause the majority of urinary tract infections (UTIs). Accurate detection of the pathogens especially in hemodialysis patients is crucial for effective treatment. The development of a promising metagenomic next-generation sequencing (mNGS) technology that enables the detection of pathogens is gaining popularity in clinical diagnosis. **Aims:** This study aims to reanalyze samples where traditional culture and commercial system identified *Klebsiella pneumoniae* and *Klebsiella aerogenes* as the pathogens by mNGS and compare the results of the two methods. **Methods**: Midstream 10 ml urine samples were collected from 150 male and female patients under more than one year of hemodialysis therapy from private laboratory and two hospitals in Baghdad and urine analysis started within 2 hours of collection. **Results**: From 150 samples 71 (47.3%) patients were positive for UTI by microscopic examination form these 59 (83.1%) were positive for UTI by urine culture and 12 (16.9%) samples produced negative urine culture. From the positive urine culture using Viteck-2 system we found that the majority of pathogenic isolates 49 (80.3%) were Gram negative bacteria the rest 12 (19.7 %) were Gram positive. The percentage of presence of *Klebsiella pneumonia* and *Enterobacter aerogenes* was 18 % and 6.55 % respectively. Four patients' samples detected with *K. pneumoniae and K. aerogenes* and three control samples tested using mNGS and the results revealed the dominance of *K. variicola and K. granulomatis.* **Conclusion**: This study showed that the majority of UTIs bacteria were gram negative, the limitations in sensitivity and accuracy of traditional detection methods compared to NGS and highlighted the importance of integrating advanced molecular techniques in diagnostic workflows.

Keywords: *Klebsiella pneumonia, Klebsiella aerogenes*, Next-Generation Sequencing.

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Introduction

After searching databases of scientific and medical literature, it was observed that the relation between urinary tract infections (UTIs) and hemodialysis therapy is inadequately researched, especially UTIs case that are caused by *K. pneumonia and Klebsiella aerogenes* (1) which are from the most relevant human pathogens, causing many types of infections worldwide, including, urinary tract, abdominal cavity, surgical sites and soft tissues infections. This Gram-negative capsulated bacterium is found within the normal flora of the mouth, skin and intestine. *Kl.aerogenes* which previously named as *Enterobacter aerogenes* is gram-negative facultative anaerobe, when the host immune system is

compromised or the mucosa is damaged, it may cause many types of infection including urinary and urogenital system infections (2).

Dialysis is a type of renal replacement therapy for the purpose of ensuring that homeostasis is maintained in those who are suffering from the loss of kidney function (3,4). Hemodialysis patients are particularly susceptible to many infections included urinary tract infections (UTIs) due to their underlying health conditions such immune disorders and frequent catheter use (5,6,7). Bacteria are responsible for the majority of UTIs cases and the accurate pathogen identification is essential for appropriate antibiotic therapy (8).

Traditionally, *K. pneumonia and K. aerogenes are* common pathogens identified in UTIs through culture methods (9). The metagenomic next-generation sequencing (mNGS) technology is gaining popularity for application in clinical diagnosis that enables pathogens detection but its applications with UTIs cases is still rather limited (10). mNGS is widely being applied in clinical laboratories for culture-independent diagnosis (11) allowing cost-effective, rapid, highly multiplexed, and accurate detection of many clinically important targets directly in clinical samples and concern has been raised over whether it could be the next common pathogen identification tool (12,13,14). This study aimed to reanalyze samples where traditional culture and commercial system identified *K. pneumoniae* and *K. aerogenes* as the pathogens by mNGS and compare the results of the two methods.

Materials and methods

Sample Collection and Processing

From November 2023 to May 2024, 150 samples of 10 ml midstream urine from hemodialysis patients were collected from two hospitals in Baghdad (The Medical City, and the Al-Kindy Teaching Hospital) and one private laboratory as shown in (Table 1).

Table (1): Distribution of samples collected from two hospitals in Baghdad and one private lab.

No.	Hospital name	count of samples
1	Private lab	82
2	Medical city	40
3	Al-Kindy teaching hospital	28
Total		**150**

Inclusion criteria

Patients who were already under more than one year of hemodialysis therapy, not under antibiotic treatment. Samples were taken before starting hemodialysis therapy.

Exclusion criteria

Samples from viral infected and under antibiotics therapy patients were excluded.

Definition of UTI

Urinalysis started in lab with dipstick then microscopic examination of the urine sedimentation, white blood cell count (40× magnification) (samples showing more than 10 white blood cells per high power field conceder as positive for UTI, cultivation of urine on Blood agar, MacConkey agar, and Mannitol Salt Agar then incubated under aerobic conditions at 37°C for 24 hours, if growth did not appear, the plates incubated for an additional 24 hours. Bacterial colonies that appeared on the plates were counted (positive urine culture was defined as detection of 10^3 cfu/ml microorganisms in the culture) (15), the pathogens identified by VITEK-2 system with its identification Cards (16,17).

Next Generation Sequencing Methodology (*16S rRNA* gene sequencing analysis).

DNA Extraction and Concentration Estimation.

The extraction of DNA was performed using the ABIOpure kit (Alliance Bio/USA) where 3 ml of urine sample centrifuged and nucleic acid extracted form pellet cells by following the kit instructions.

Quantus fluorometer system (Promega/USA) used to estimate the DNA concentration. 1 µl of template DNA with 200 µl of Quantifluor diluted dye was mixed. After incubation at room temperature for 5 min, DNA concentration values were detected.

Primer preparation

The primers used in our study supplied by Macrogen Company in lyophilized form (Table 2).

Lyophilized primers were dissolved using nuclease-free water to give a final concentration of 100 pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10 µl of primer stock solution to 90 µl of nuclease-free water to obtain a working primer solution of 10 pmol/µl.

Preparation of DNA library

16S ribosomal RNA libraries were amplified by PCR. Primers target the *16S rRNA* gene hypervariable V3 and V4 regions with the overhang adapter sequences (18). The composition of the reaction mixture and the thermal amplification profiles were presented in the (Table 3). Libraries were prepared following the protocol for preparing *16S Ribosomal RNA* gene Amplicons identified by in iSeq 100 Sequencing System (Illumina/USA).

Table (2): The sequences of primers used in this study (19).

No.	Primer name	Sequence	Product size bp	Company/Origin
1	16S F	5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG -3′	550	Macrogen, Korea
2	16S R	5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -GACTACHVGGGTATCTAATCC-3′		

Table (3): PCR contents and program for library preparation.

Reaction Component	Volume (µl)	Thermal amplification profile
Molecular grade water	0	• 95°C for 3 minutes • 25cycles of: - 95°Cfor 30 seconds - 55°Cfor 30 seconds - 72°Cfor 30 seconds • 72°Cfor 5 minutes • Hold at 4°C
GaTaq Green Master Mix	12.5	
16SampliconPCR forward primer	1 (10 µM)	
16SampliconPCR reverse primer	1 (10 µM)	
DNA	10.5	

Agarose gel casting

The agarose solution (2%) was poured into the gel tray after, and it left to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray filled with 1 X (Tris-acetate-EDTA) TAE-electrophoresis buffer (Promega/USA) until it reached 3-5 mm over the gel's surface. PCR products (5µl) were loaded directly to wells. Electrical power was turned on at 100v/mAmp for 60min. The Ethidium bromide (Promega/USA) -stained bands in gel were visualized using gel imaging system (20).

PCR CleanUp 1

In this step, the ProNex® Size-Selective Purification System (Promega / US) was used to purify the 16S V3 and V4 amplicon away from free primers and primer dimers. The steps of PCR Clean-Up illustrated in figure (1).

Index PCR

The Nextera XT Index Kit (Illumina/USA) was used to attach dual indices (Table 4).

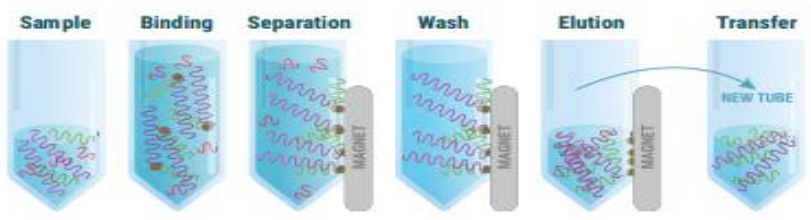

Figure (1): Size-selective purification of ds-DNA fragments larger than a given size.

Table (4): Index PCR reaction.

Component	Volume (µl)
DNA	5
Nextera XT Index Primer 1 (N7xx)	5
Nextera XT Index Primer 2 (S5xx)	5
2x KAPA HiFi HotStart ReadyMix	25
PCR Grade water	10
Total	50

PCR Clean-Up 2

The same protocol was used in the first PCR CleanUp performed using ProNex® Size-Selective Purification System (Promega / US) following the kit instructions.

Library Quantification and Pooling

The constructed libraries concentration was assessed by fluorometric quantification method used dsDNA-binding dyes to quantify libraries flowing the Quantus Fluorometer system instruction.

Results

UTI and Culture Findings

The results showed that out of 150 urine samples, 71 (47.3%) were positive for UTI according to microscopic examination. Culture media results showed that: 59 (83.1%) samples from which were positive by microscopic examination gave positive urine culture and 12 (16.9%) samples produced negative urine culture. From 59 positive urine culture we identified 61 bacteria isolates. 49 (80.3%), of the identified bacteria were Gram-negative. *Klebsiella pneumonia* and *Enterobacter aerogenes* were the identified pathogenic bacteria in 11 (18 %) and 4 (6.55 %) samples respectively.

Next-generation sequencing

Amplicons pooled in equimolar concentrations of 100 pM for iSeq sequencing according to the manufacturer's recommendations. Sequencing was performed using iSeq 100 i1 Reagent v2 (300- cycles) and iSeq 100 Sequencing System (Illumina/USA).

Data Analysis

16S MetagenomicsApp in BaseSpace Sequence Hub used for Sequencing results analyses.

Library preparation and mNGS Findings

To gain insight into potential urinary pathogenic bacteria, we analyzed 4 of clinical urine samples for their bacterial content using *16S rRNA* gene signatures, where form 7 (4 different patients' samples and 3 different control samples collected from healthy non infected persons) DNA template next generation sequencing data was successfully generated. Electrophoresis process results of the library preparation products as in figure (2).

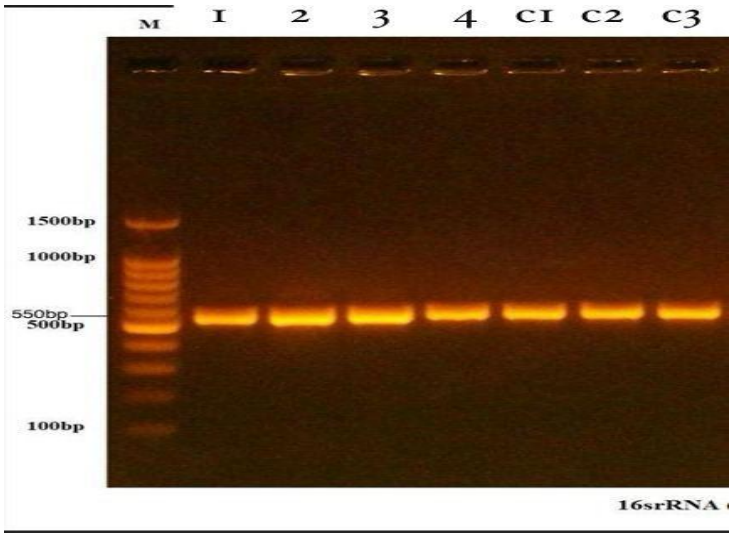

Figure (2): The amplification results of unknown bacterial species *16s RNA* gene on 2% agarose gel electrophoresis stained with Eth.Br. DNA Marker: 100bp ladder marker. Lanes resemble 550bp: PCR products.

The culture results for sample number one revealed the pathogenic bacteria to be *K. pneumonia*. The NGS results showed the presence of this species only in (1.62) accompanied by the dominance of *K. variicola* (30.87%), *K. granulomatis* (21.74%), and other *K. spp*. 6.5%. The control sample contains these species as following: *K. pneumonia* (C1:0, C2:0, C3:0.01%), *K. variicola* (C1:0.01, C2:0, C3:0.1%), *K. granulomatis* (C1:0.07, C2:0.02, C3:0.04%).

Sample number two culture results indicated that the pathogenic bacteria was *Enterobacter aerogenes* (recently renamed as *K. aerogenes* (21,22) the NGS results showed presence of (with the percentage of their presence): *K.variicola* (26.65%) which is Gram-negative, facultative anaerobic opportunistic pathogen bacteria responsible for infections including tract urinary tract infections (23), *K. granulomatis* (8.75%) which cannot be cultivable on routine culture media as many studies mentioned and can causes sexually transmitted disease and urinary tract infection(24) (25), *K. spp* (1.43%), *K. pneumonia* (0.134%) which are the most common UTI causative bacteria, as many studies mentioned, like the study of Sokhn *et al.* in Beirut in 2020 (26).

In sample number three, culture results indicated the pathogenic agent was *K.pneumonia*, but when we looked at the results of NGS, we found the presence of *Gardnerella vaginalis* (69.25%) with the highest rate of presence among the other members of the sample bacterial community, which is facultative anaerobic bacteria related to the UTI (27), *Desulfovibrio tunisiensis* (3.61%), strictly anaerobic, that can be implicated in infections, especially in immunocompromised individuals like hemodialysis patients as previous studies showed (28) (29). *K. variicola* presence in only 0.71%. Despite the presence of *Klebsiella spp*. and the existence of research suggesting their potential to cause infection, our belief is that *Gardnerella vaginalis* and *Desulfovibrio tunisiensis*, which had the highest incidence rate within the patient sample and were absent in the control samples, were the cause of the infection. On the other hand, the presence of *K. spp*. occurred in the control samples in small and

did not give any symptoms of urinary tract infection. Following the percentage of presence of these species within the control sample: *Gardnerella vaginalis* (C1:0.1, C2:0.01, C3:0.0%), *Desulfovibrio tunisiensis* (zero in all control samples), and *K. variicola* (C1:0.01, C2:0, C3:0.1%).

The culture results for sample number four indicated that the pathogenic agent was a mix of gram-negative bacteria, *Enterobacter aerogenes* (recently renamed as *K. aerogenes* (21) and gram-positive bacteria, *Staphylococcus haemolyticus.* However, when we analyzed the data from NGS, we found *K. granulomatis* (26.6%). *Klebsiella variicola* (13.4%) *K. pneumonia* (2.2%). *Staphylococcus haemolyticus* was absent from NGS results (Table 5).

Table (5): Percentage of bacterial identification in patients and control samples according to mNGS data results.

Sample		Culture results	*Gardnerella vaginalis %*	*Desulfovibrio tunisiensis %*	*Klebsiella variicola %*	*Klebsiella granulomatis %*	*Klebsiella aerogenes %*	*Klebsiella pneumonia %*
Patients	1	Positive	0.33	0.017	30.87	21.74	0.0	1.62
	2	Positive	0.60	0.063	26.65	8.75	0.0	0.134
	3	Positive	69.2	3.6	0.71	0.3	0.0	0.0
	4	Positive	0.05	0.0	13.3	26.6	0.0	2.2
Control	1	Negative	0.018	0.0	0.013	0.07	0.0	0.0
	2	Negative	0.017	0.0	0.0	0.02	0.0	0.0
	3	Negative	0.006	0.0	0.19	0.04	0.0	0.012

Discussion

The findings of this study confirmed the wide spreading of Gram-negative bacteria in compared to the Gram-positive bacteria due to their outer membrane contains lipopolysaccharides (LPS), which can act as endotoxins and help these bacteria evade the host's immune system. The outer membrane containing efflux pumps that acts as a barrier against antibiotics and immune system components (29).

Gram-negative can form biofilms, helping to adhere to surfaces and are difficult for both the host immune system and antibiotics to penetrate. Gram-negative bacteria often produce a variety of virulence factors, such as exotoxins and enzymes that can disrupt host tissues and evade immune responses.

Genetically Gram-negative bacteria have higher genetic plasticity that allows them to acquire resistance genes and adapt to different environments more rapidly than Gram-positive bacteria (30, 31). Our results were similar to Naqid *et al,.* study results which conducted in Iraq 2020 (32).

On the other side the molecular part results showed differences between traditional culture methods which are reliable and widely used and NGS in

detecting *K. variicola* and *K. granulomatis* demonstrates the limitations of conventional diagnostic approaches where culture methods less sensitive to certain pathogens and failed to differentiate between closely related species, identified only one or few of infectious bacterial types in each sample (31). In contrast the NGS had a significantly higher detection rate for infectious bacterial types and mixed infection and more accurate method since the isolates were different from those identified by traditional method, these results were similar to the results founded by Duan, *et al.,* in study conducted in China in 2022, where the findings of mNGS was higher in significant manner than culture medium in total positive rate (100.0% vs. 31.6%) (33).

Klebsiella variicola and K. granulomatis is not in the databases of Vitek-2 and commercial systems so currently, isolates may be incorrectly identified as *K. pneumoniae* (34). This misidentification was particularly problematic in clinical and microbiological settings where accurate identification is crucial for appropriate treatment and epidemiological tracking (31). The identification of *K. variicola and K. granulomatis* by NGS underscores the limitations of culture-based methods, which may fail to distinguish between closely related species or less common pathogens. This can impact treatment decisions and patient outcomes, as *K. variicola and K. granulomatis* may have different antibiotic susceptibility profiles compared to *K. pneumoniae* and *K. aerogenes.* The discrepancy between traditional culture methods and NGS in detecting *K. variicola* and *K. granulomatis* demonstrates the limitations of conventional diagnostic approaches. NGS exhibits a higher probability of identifying pathogens with increased specificity and accuracy in comparison to the traditional culture technique which is similar to Zhao *et al*., (2024) results (35).

Conclusions:

Based on the gained results, we found that NGS gave a higher probability of identification of pathogens with increased accuracy and specificity in comparison to the culture traditional technique. NGS offers a more comprehensive and accurate identification by analyzing the entire microbial DNA present in a sample. This case emphasizes the need for integrating NGS into routine diagnostic practices to enhance pathogen detection, ensure appropriate treatment as well as to developing and validating protocols that combine traditional and molecular methods to improve diagnostic accuracy. Additionally, increasing awareness of *K. variicola and K. granulomatis* and its clinical significance is essential for better management of UTIs in hemodialysis patients.

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